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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INFORMATION

Leishmania *spp*. are obligated intracellular protozoa of the genus Leishmania belonging to the Trypanosomatidae family (Margulis et al. 1996) that cause different clinical forms of leishmaniasis in humans and represent an important public health problem in many countries all over the world. This parasite is named after W.B. Leishman, who described it in 1901. This unicellular organism has a particular organelle called 'kinetoplast' – the main characteristic of the Kinetoplastidae order – which is located at the basal position next to the origin of the flagellum, and has mitochondrial DNA. There is no evidence of obligated sexual reproduction; they essentially multiply by binary fission and have a digenetic cycle within an invertebrate organism (vector) and a vertebrate organism (mainly mammals). There are more than 20 species and subspecies of *Leishmania* that have been identified as infective for humans, and more species are emerging, especially in association with HIV/AIDS. Thirty species of sand fly have been identified as vectors for the parasite.

1. LEISHMANIASIS

Leishmania *spp.* is an endemic parasite which can be found on four continents. The disease leishmaniasis derived from it is associated with different symptoms, including fever, malaise, weight loss, anaemia, swelling of the spleen, liver, and lymph nodes, and a variety of clinical forms that range from simple, self-healing skin ulcers to a severe life-threatening disease: visceral leishmaniasis. According to the World Health Organization (WHO), leishmaniasis is considered one of the six most important tropical diseases in the developing countries.
The leishmaniases can be classified into four main clinical forms:

1.1. CLINICAL FORMS

1. **Visceral leishmaniasis** *(VL),* also known as kala-azar, is the most severe form of the disease and fatal if left untreated. The species responsible for this disease are *L. donovani* and *L. infantum* in the Old World and *L. chagasi* (synonymous *L. infantum*) in the New World. The incubation period varies from ten days to one year with an average of two to four months. The parasites transmitted after the sandfly's bite infect the macrophages of the spleen, liver, gut and bone marrow. In general, after six or twelve months an opportunistic infection may appear, produced by other pathogens that can cause tuberculosis or pneumonia (Fig. 1a).
2. **Cutaneous leishmaniasis** *(CL)* causes 1–200 simple skin lesions which self-heal within a few months or years but which leave disfiguring scars ("Uta" due to *L. (V.) peruviana* in South America, Baghdad ulcer, Delhi boil or Oriental sore, due to infection with *L. major* in Africa and Asia). This is the most common form of leishmaniasis, representing 50–75% of all new cases (Fig. 1b).

3. **Mucocutaneous leishmaniasis** *(MCL)* is a mutilating disease due to *L. (V.) braziliensis* infection. It begins with skin ulcers after one or four months of the vector's bite, and starts to heal after six or fifteen months of the infection. However, there is a second lesion that appears by metastasis, causing severe and massive tissue destruction, especially of the nose, mouth and throat cavities. Normally, a second infection of bacterial origin could appear under these conditions. The disease may be life-threatening if complications in the respiratory system occur (Fig. 1c).

4. **Diffuse cutaneous leishmaniasis** *(DCL)* produces disseminated and chronic skin lesions resembling those of leprotamous leprosy. It is a difficult to treat and disabling disease (Fig. 1d).

Fig. 1a *Visceral leishmaniasis (http://www.who.int/leishmaniasis/en/)*

Fig. 1b *Cutaneous leishmaniasis (Photo provided by the "Grupo de Estudios de leishmaniasis" IMTAvH-UPCH)*

Fig. 1c *Mucocutaneous leishmaniasis (Photo provided by the "Grupo de Estudios de leishmaniasis" IMTAvH-UPCH)*

Fig. 1d *Diffuse cutaneous lLeishmaniasis (http://www.who.int/leishmaniasis/en/)*

In general, the clinical manifestations result from a complex series of interactions between the parasite (tropism and virulence) and the human hosts' immune response (Liew and O'Donnell 1993; Murray et al. 2005). Although most of the information on the immunologic mechanisms upon infection and protection from the Leishmania parasites were accumulated from studies in mice, some critical findings of murine CL have been confirmed in humans in recent years. The immune response to VL and the pathogenesis of the disease in humans deviates considerably from the murine models (Saha et al. 2006; Silveira et al. 2004).

Cell-mediated immune response (CMI) plays a major role in the susceptibility (CD4+ Th2) or resistance (CD4+ Th1) to the disease. Severe manifestation of CL (MCL and DCL) is associated with a strong Th2 compared to a predominant Th1 response in mild manifestations of the disease (Gaafar et al. 1995). On the other hand, it is well documented that VL is characterized by suppression of CMI, as proven by patients unresponsiveness to the Leishmanin skin test (LST) or Montenegro test, measuring a delayed-type hypersensitivity (DTH) reaction to an intradermal injection of Leishmanial antigens (Manson-Bahr 1961).

Humoral immune response cannot be ruled out as VL is marked by high levels of Leishmania-specific antibodies, which appear soon after infection and before the development of cellular immunologic abnormalities. The exact role of these elevated antibodies in resolving of the disease and generating protective immunity is unknown. In CL, low levels of antibodies are usually present during the active phase of the disease; however, in some studies the presence of antibodies against *L. (V.) braziliensis* infection in the sera of infected patients has been critically monitored and utilized for the diagnosis and prognosis of the disease (Montoya et al. 1997; Valli et al. 1999; Brito et al. 2000; Brito et al. 2001; Romero et al. 2005).

Depending on the Leishmania species responsible for the infection and the patient's cell-mediated immune response (Th1/Th2 ratio), different clinical forms of the aforementioned diseases will develop.

1.2. DIAGNOSIS

The "golden standard" method for diagnosing leishmaniasis is the microscopic examination of Giemsa-stained slides prepared from biopsies, scrapings, or impression smears. The aim of this method is to identify amastigotes – the intracellular form of the parasite – according to their characteristics: size (2-4 μm), round to oval shape and internal organelles, nucleus and kinetoplast (Herwaldt 1999). Other traditional methods are the *in vitro* culture of infected tissues or lesion aspirates, or *in vivo* infections using animal models.

As it sometimes proves difficult to obtain tissue samples in patients suffering from VL, the following serological methods are mainly used: the freeze-dried agglutination test (DAT) to detect antibodies; the rK39 dipstick based on recombinant leishmanial antigen of *L. infantum*, which show a high sensitivity (67–100%) and specificity (93–100%); and a latex agglutination urine test to detect antigens, which represents a promising alternative to be used in HIV co-infected cases (Report on Leihsmaniasis, TDR/SWG/04).

DNA-based methods, like PCR, usually have high sensitivity and specificity, but require sophisticated equipment and highly trained personnel that are not currently available in peripheral laboratories of most developing countries. As a result, additional assessment of its field applicability is still needed.

1.3. TREATMENT

Leishmania *spp.* has different sensitivity to the drugs developed so far. Furthermore, visceral and cutaneous sites of infection call for different pharmacokinetic requirements of the drugs to be used. As a result of the toxic side effects and resistance to the main drug treatment, pentavalent antimonials, in Bihar (India) (Lira et al. 1999), elimination of this disease will be difficult. In addition, the presence of an animal reservoir for some species also plays a major role.

CL is treated to accelerate cure, reduce scarring and prevent dissemination (mucosal disease) or relapse (Murray et al. 2005). It is generally treated with parenteral antimony for 20 days, while a 28-day course is the standard regimen for MCL. In VL, antimony remains the main therapeutic treatment in all regions with the exception of the Indian subcontinent. In Southern Europe, where the cure rate of antimony is 90%, most patients are now receiving liposomal Amphotericine B treatment, which also has good efficacy for cutaneous and mucosal leishmaniasis (Berman 2003). Amphotericine B is a very expensive product to be introduced in other endemic countries (mainly developing countries). The most recent treatment introduced for VL is the Miltefosine (alkylphosphocholine), a drug originally developed as an anticancer drug. In March 2002, Miltefosine was registered in India for oral treatment of VL in a 28-day course. Even though the high efficacy shown, some of its limitations are its toxicity in children and in patients with immunosuppression, and its teratogenic potential which excludes its use in women of child-bearing age (Croft and Coombs 2003). Paromomycin (aminosidine), an antibiotic agent, also has antileishmanial activity and has been used in several clinical trials for cutaneous and visceral leishmaniasis (Croft and Coombs 2003). The group of antifungal drugs with antileishmanial activity, the azoles (e.g., fluconazole, ketoconazole, itraconazole) have been tested in several trials for cutaneous and visceral leishmaniasis with differing results. A number of other products derived from plants have shown promising results in experimental models but should be considered with caution if applied in humans.

Among patients who are also infected with human immune deficiency virus (HIV), slow response to treatment and a high rate of relapse (around 60% in the first year) for different drugs used are the main problems to be addressed (Laguna et al. 1994, Alvar et al. 1997, Russo et al. 2003).

1.4. TRANSMISSION

The vectors of leishmaniasis are female sandflies – tiny (about 2-3mm long) sand-colored blood-feeding flies that breed in forest areas, caves, or the burrows of small rodents. Wild animals (such as rodents) and domesticated animals (such as dogs) as well as humans

themselves can act as reservoirs of infection. The sandfly vector is usually infected with one species of *Leishmania* by ingesting blood from infected reservoir hosts.
Approximately 30 species or subspecies of sandfly are proven vectors, and probably more than 40 additional species are involved in transmission (Reithinger et al. 2007).
Old World forms of Leishmania are transmitted by sandflies of the genus *Phlebotomu*s (Europe, North Africa, Middle East and Asia), while New World forms are mainly transmitted by flies of the genus *Lutzomyia* (from Southern USA to Northern Argentina). Sandflies are relatively weak, noiseless fliers; they rest in dark, moist places, and are typically more active during the evening (Herwaldt 1999, Killick-Kendrick 1999).
Most leishmaniases are zoonotic (animals are reservoirs which maintain and disseminate the Leishmania parasites), and humans become infected only when accidentally exposed to the natural transmission cycle. Most of the well-recognized Leishmania species known to infect humans are zoonotic, which include agents of visceral, cutaneous and mucocutaneous forms of the disease in both the Old (*L. (L.) major*) and New World (*L. (V.) braziliensis* and *L. (L.) mexicana* complexes). However, for the anthroponotic forms (those transmitted from human to human through the sandfly vector, mainly associated with *L. tropica*), humans are the sole reservoir host (Desjeux 2001). A recent group of risk factors that also contribute to increased Leishmania transmission includes urbanization and deforestation, economic hardship, natural disasters, armed conflicts and tourism (WHO 2002; Reithinger et al. 2007).
Other forms of Leishmania are congenital (Eltoum et al. 1992; Figueiro-Filho et al. 2004) and transmitted parenterally (through blood transfusion, needle sharing and laboratory accident) (Bruce-Chwatt 1972; Dejeux and Alvar 2003).

1.5. CONTROL

The main control strategy in most endemic countries is the detection and treatment of active cases. Another method, if feasible, is the vector control programme based on infrequent and sporadic spraying of houses with insecticides (pyrethroids). Treated bednets is yet another protection alternative; sandflies are endophagic and most active when people are asleep. However, as these bednets require re-impregnation, long-lasting insecticide-treated bednets would be the solution, but these are expensive and the distribution to the endemic countries is quite a challenge (Murray 2005).
With respect to VL – primarily zoonotic – the main control measure is to target the animal reservoir, for example, in the case of dogs to avoid canine leishmaniasis by dipping dogs in insecticide, applying topical lotions and introducing deltamethrin-treated collars for dogs to protect them from sandfly bites (Davis et al. 2003; Desjeux 2004).
There is no vaccine against any form of leishmaniasis for general human use, despite the available information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccine candidates that generate protection in experimental animals. The parasite's complexity plays a major role in this lack of vaccine, while another reason could be the potential market to be addressed: mainly poor people from developing countries, who do not constitute an attractive market to many of the pharmaceutical companies (Khamesipour et al. 2006). However, a number

of laboratory efforts are underway (new antigens and adjuvants, live-attenuated vaccine, recombinant proteins, DNA vaccines, bacterial expressing leishmanial antigens, and targeting dendritic cells) (Murray et al. 2005; Khamesipour et al. 2006).

1.6. LIFE CYCLE OF THE PARASITE

All Leishmania species are transmitted by the sandfly vector from the genus *Phlebotomus* (species from the Old World) or the genus *Lutzomia* (species from the New World) and it is generally accepted that they are obligated intracellular parasites in the macrophages from the mammal hosts (Blackwell and Alexander 1983).
During its life cycle, both in the mammal host and in the sandfly vector, the Leishmania parasite comes in different morphological and biological phenotypes/forms, which include the promastigote appears as stage with variable morphology located inside the gut of the female sandfly (long flagellated parasite) and the amastigote stage (round form without free flagellum) inside the phagolysosome of the mammal host (Killick-Kendrick 1990). These phenotypes rely on variations in the expression of (specific) genes that allow the parasite to survive in these two different environments. The more interesting parasite forms in terms of virulence and pathogenicity are the metacyclic promastigotes, responsible for initiating the infection, and the amastigotes forms, which are in charge of maintaining the infection in the mammal host (Fig. 2a, 2b and Fig. 3).

Fig. 2a *Metacyclic promastigotes (in vitro culture)**

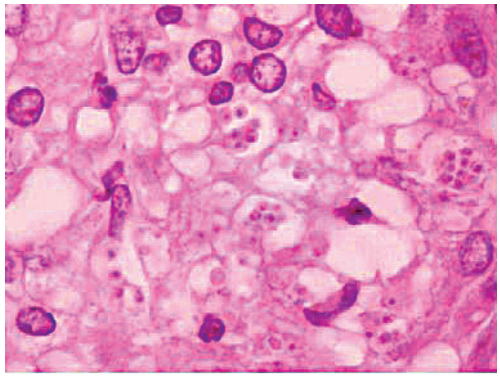

Fig. 2b *Amastigotes (in vivo infection)**

*Photos from the thesis experimental procedures.

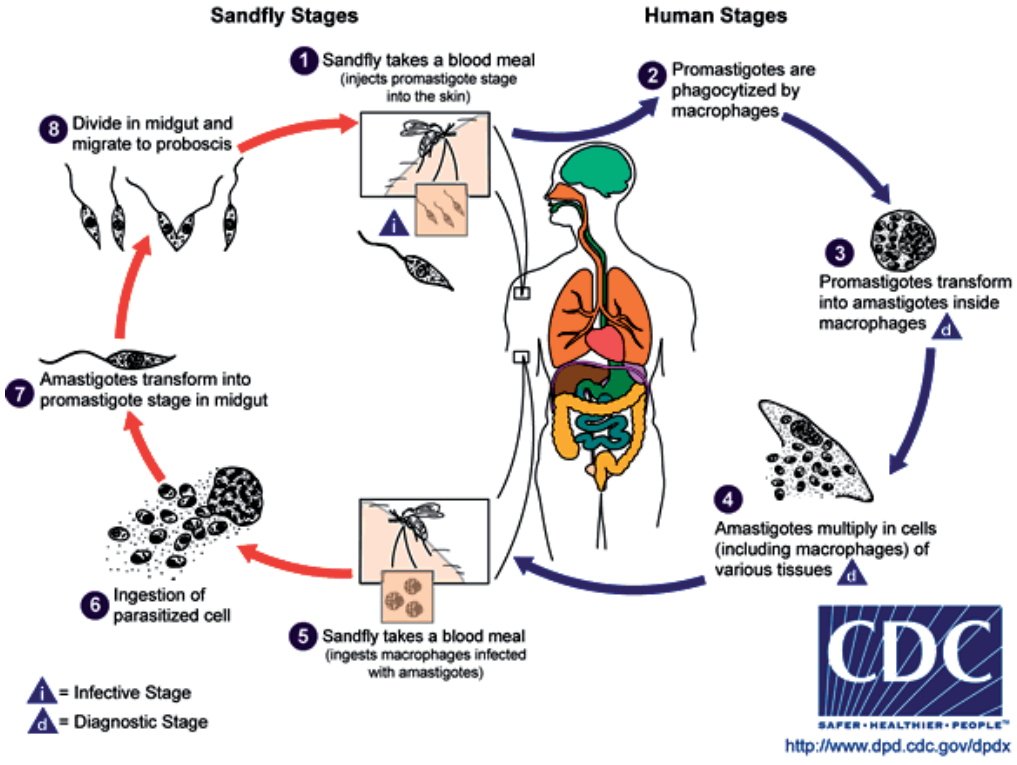

Fig. 3 *Leishmania life cycle (http://www.dpd.cdc.gov/dpdx /HTML/leishmaniasis.htm)*

Promastigote parasites are injected into the mammalian host when the sandfly takes a blood meal. In the vertebrate host, parasites are quickly taken up by tissue phagocytes, monocytes and neutrophils, which were attracted to the biting site due to the damage caused by the sandfly (Wilson et al. 1987). Within the macrophage, the parasite loses its flagellum and transforms into a non-motile amastigote form. This amastigote is capable of surviving and replicating in the very acidic environment of the phagolysosome. If the amastigote remains localized at the inoculation site, different cytokines are released and all reactions are generated resulting in the development of CL (Barral-Netto et al. 1995). In the case of VL, the expression of newly acquired infection varies from none (subclinical), to oligosymptomatic, to fully established (kala-azar), spreading to the organs of the mononuclear phagocytic system (Murray et al. 2005). In other cases, amastigotes may also spread to other cutaneous sites other than the inoculation site; this is called DCL. If they also migrate to the mucosa, MCL appears.

When a sandfly takes a blood meal from an infected host, it acquires either free amastigotes or amastigote-infected macrophages. Within the sandfly, amastigotes undergo several divisions and progressive metabolic and morphologic changes to long slender nectomonads (procyclic forms: short, ovoide, slightly motile). Non-infective promastigotes, which are continuously dividing, attach to the microvilli of the thoracic midgut through lipophosphoglycan (LPG) and transform into infective promastigote with shorter body, longer flagellum, non-replicating forms, rapidly moving (metacyclic forms), located

in the anterior midgut and foregut. These infective promastigote forms will be injected into the mammalian host during the next blood meal (Sacks 1989).

Axenic cultures: effect of temperature and pH

Differentiation of the parasite is mainly induced by changes in temperature and pH of the extracellular environment. In addition to the morphological changes, this stress also produces various modifications at the metabolic level to ensure the complete differentiation, showing the parasite's high adaptation capability.

Temperature effect: The temperature found by the parasite inside the macrophages differs from that at the inoculation site (32–37°C). The thermotolerance of the strains producing VL is even bigger than that of the other strains. While *L. donovani* can resist a thermal shock of 39°C *L. major* cannot survive in this temperature. It was also demonstrated *in vivo* that the infectivity increases if the parasites were submitted to a thermal shock before they were inoculated into an animal (Zilberstein and Shapira 1994). It is possible that this kind of stress increases the quantity of coding transcripts from the *hsp* (heat shock protein) gene family, like *hsp70* and *hsp83* (Zilka et al. 2001).

The pH influence: While the intracellular pH of the parasite ranges from 6.8 to 7.4 between the two life stages, the extracellular pH varies between 7.5 and 4.5. Some enzymatic experiments have shown variations in the optimal pH for many of the enzymes in the two life stages (Zilberstein and Shapira 1994). The *gp63* protein exists in the two parasite forms, so its activity is adapted to the pH of the environment. Though one study (Garlapati et al. 1999) shows that the acidic pH contributes to stabilization of the *hsp83* transcript at 26°C, another study did not find any augmentation on the translation because of thermal shock (Argaman et al. 1994).

1.7. TAXONOMY

The Leishmania genus belongs to the Trypanosomatidae family of the Kinetoplastidae order, which consists of a set of organisms characterized by the presence of kinetoplast, an organelle corresponding to a complex network of concatenated DNA circles (Chen et al. 1995).

Classification of the genus Leishmania (after Levine et al. 1980)

This classification was suggested by Lainson & Shaw, 1987.

Kingdom:	Protista (Haeckel, 1866).
Subkingdom:	Protozoa (Goldfuss, 1817).
Phylum:	Sarcomastigophora (Honigberg & Balamuth, 1963).
Subphylum:	Mastigophora (Deising, 1866).
Class:	Zoomastigophorea (Calkins, 1909).
Order:	Kinetoplastida (Honigberg, 1963, emend. Vickerman, 1976).
Suborder:	Trypanosomatina (Kent, 1880).
Family:	Trypanosomatidae (Doflein, 1901, emend. Grobben, 1905).
Genus:	Leishmania (Ross, 1903).

The classification and identification of the different Leishmania species has constantly changed since the discovery of these parasites. The early classifications were based on clinical and epidemiological characteristics of the disease they produce in humans, on the biological and morphological characteristics of the parasite inside the vector, animal models and *in vitro* cultures, and on geographical distribution. Nowadays, following the development of new techniques, the Leishmania parasites are classified according to their molecular, biochemical and immunological characteristics. The most useful techniques include those based on DNA, like RAPD (random amplified polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), PCR (Polymerase Chain Reaction) and PFGE (Pulse Field Gel Electrophoresis). Multilocus Enzyme Electrophoresis (MLEE) remains the current golden standard technique that allows the genotypification based on markers that are able to detect both alleles in this diploid organism. MLEE was used in the majority of population and epidemiological studies on Leishmania (Schonian et al. 2001, Bañuls et al. 2002).

So far, the number of infective species described is approximately 30 for mammals and 17 for reptiles. The species that infect mammals have been further divided into two subgenera: (i) the species that develop in the vector's anterior and midgut belong to the subgenus Leishmania (they constitute the majority and are present in both the Old and New World) and (ii) the species that continue to develop in the posterior gut belong to the subgenus *Viannia* (such as *L. (V.) braziliensis*; these are only found in the New World). Leishmania from reptiles has been grouped within the subgenus Sauroleishmania, restricted only to the Old World (Stevens et al. 2001).

Cupolillo et al. (2000) proposed to separate the genus Leishmania into two subdivisions, Euleishmania and Paraleishmania, called sections by analogy with a similar division in the genus Trypanosoma. Euleishmania would include the subgenera Leishmania and *Viannia*; the section Paraleishmania would include all the species whose classification is uncertain, such as *L. herreri*, *L. equatoriensis*, *L. deanei*, *L. hertigi* and *L. colombiensis*, as well as some species and strains from the genus Endotrypanum.

Some aspects of species classification are still subject to controversy due to different factors, including the presence of hybrids like *L. (V.) panamensis* and *L. (V.) braziliensis* (Belli et al. 1994), *L. (V.) braziliensis* and *L. panamensis/guyanensis* (Bañuls et al. 1997),

L. major and *L. arabica* (Kelly et al. 1991), or *L. (V.) braziliensis* and *L. (V.) peruviana* (Dujardin et al. 1995a). In addition, the genetic differentiation between some species initially described on the basis of different geographical origins, for example *L. (V.) panamensis* and *L. (V.) guyanensis*, is contested (Bañuls et al. 1999).
Finally, the link between clinical manifestations and taxonomic species is not simple, as a single species can cause different clinical outcomes (*L. (V.) braziliensis*: cutaneous and mucocutaneous forms) whereas different species can sometimes cause similar outcomes (*L. (V.) braziliensis* and *L. (V.) guyanensis*).

1.8. EPIDEMIOLOGY

Leishmaniasis is widespread in 88 countries on 4 continents, 22 in the New World (from northern Argentina to southern Texas; but not in Uruguay, Chile or Canada) and 66 in the Old World (Asia (except South-east Asia, where it does not exist), the Middle East and Africa (particularly East and North Africa, with sporadic cases elsewhere)). There are also reports of human infections in 16 countries in Southern Europe, including France, Italy, Greece, Malta, Spain and Portugal. The disease has not been reported in Australia or Oceania (WHO 2004, Herwaldt 1999) (Fig. 4).
More than 90% of CL cases occur in Iran, Afghanistan, Algeria, Syria, Saudi Arabia, Brazil and Peru. 90% of all cases of MCL are found in Bolivia, Brazil and Peru. More than 90% of VL cases occur in Bangladesh, Brazil, India, Nepal and Sudan.

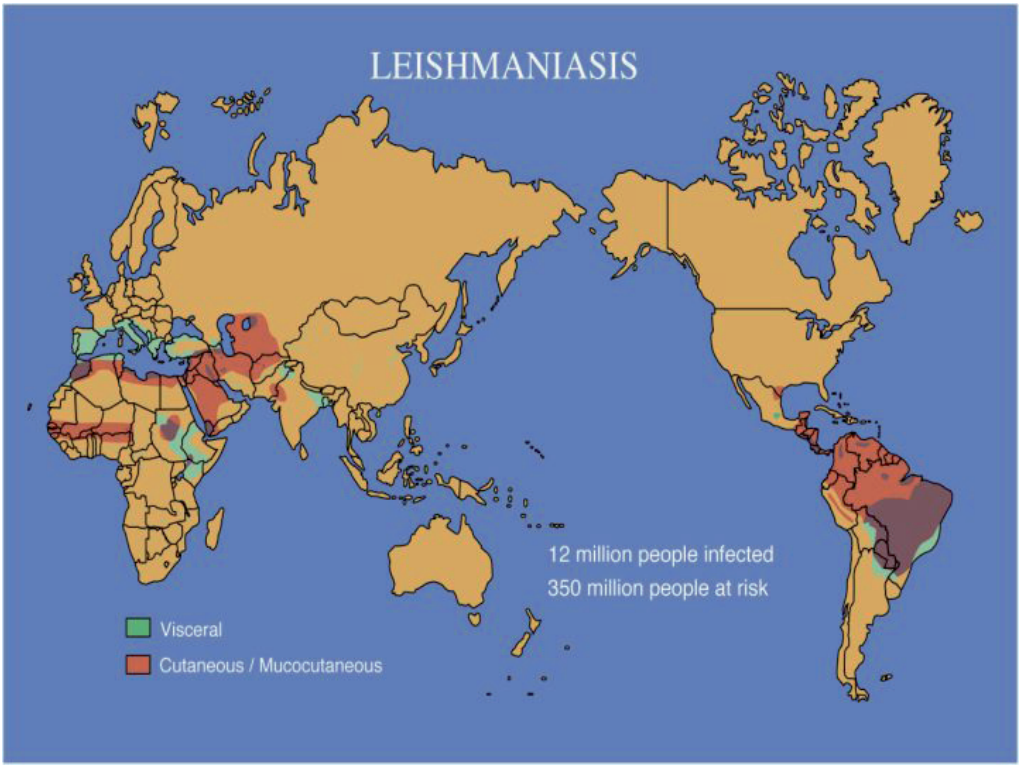

Fig. 4 *Map of worldwide leishmania distribution (from Handman 2001)*

According to the geographical distribution, leishmaniasis is mainly associated with poverty (except in Europe) and perceived as a low-priority problem by governments, society and, in some cases, patients themselves. Governments are facing other urgent health problems (in terms of greater magnitude or impact) or do not have enough resources. The leishmaniasis cases occur mainly in the peripheral areas, far from the central governments. As a result, detailed information about the importance and magnitude of the problem and measures of interventions and control measures are lacking. The strategy of the majority of governments mainly consists of haphazardly responding to epidemics rather than anticipating and preventing the disease. The population living in the risk areas have no information about the importance of prevention, diagnosis and treatment (WHO 2004).
Disability Adjusted Life Years (DALY) due to leishmaniasis is close to 2.4 million, there are 12 million of cases and 70,000 deaths annually, and 350 million people are living in risk areas. The annual incidence of CL+MCL+DCL is 1.5 millions and 500,000 for VL (WHO 2004). The real numbers are probably higher than those mentioned in the official reports from the endemic countries, because reporting the disease is compulsory in only 32 countries in a passive detection system and reports do not include data from private clinics and non-governmental organizations.
Even though leishmaniasis is now considered one of the most neglected diseases, there is a clear and alarming increase in the number of cases in several regions. For example, the incidence of CL in Brazil increased from 21,800 cases in 1998 to 60,000 cases in 2003; from 14,200 cases in 1994 to 67,500 in 2002 in Kabul (Afghanistan); and from 3,900 cases in 1998 to 6,275 in 2002 in Aleppo (Syria) (Desjeux 2004). The main reasons associated with these increases are mainly man-made (development of new settlements, intrusion into primary forest, deforestation, massive migration from rural to urban areas, fast and unplanned urbanization, and the building of dams, wells and new irrigation schemes that bring non-immune urban dwellers into endemic rural areas) and natural environmental changes. However, individual risk factors such as malnutrition and more recently immunosuppression owing to HIV co-infection also play an important role, as well as the emergence of antileishmanial drug resistance. Intravenous drug users have been identified as the main population at risk of Leishmania/ HIV co-infections in Europe, while in Africa and India, migrants, seasonal workers, refugees, sex workers and truck drivers are the target population (WHO 2002).
The geographical distribution of leishmaniasis is also related to the distribution of the sandfly species, its susceptibility to cold climates, its tendency to take blood from humans or animals only, and its capacity to support the internal development of specific Leishmania species (Table 1).

Table 1: Species Geographic Distribution (modified from Herwaldt B. Lancet 1999)

Cutaneous leishmaniasis			
Old World	Country or Region	Vector	Reservoir
L. aethiopica	Ethiopy and Kenya	*P. longipes, P. pedifer*	Hyraxes
L. major	Africa and Asia	*P. papatasi, P. buboscqi*	Gerbils, Rodents
L. tropica	Europe, Asia and North Africa	*P. sergenti*	Humans
L. donovani	North-East India, Bangladesh, Burma	*P.argentipes*	Humans
L. infantum	Mediterranean basin, Middle East, China, Central Asia	*P. perniciosus, P. ariasi*	Dog, foxes, jackals
New World			
L. mexicana complex: L.amazonensis	Central and South America	*Lu. olmeca*	Forest rodents
Viannia Subgenus:			
L. (V.) braziliensis	Tropical forest of South and Central America	*Lutzomia* spp. *Psychodopygus wellcomei*	Forest rodents, peridomestic animals
L. (V.) guyanensis	Guyana, Surinam, Brazil	*Lu. umbratilis*	Sloths (Chleopus), Arboreal anteaters (Tamandua)
L. (V.) peruviana	West Andes of Peru, Argentine highlands	*Lutzomia* spp.	Dogs
L. chagasi (synonymous of *L. infantum*)	South America	*Lu. longipalpis*	Foxes, dogs, opossums

2. AMERICAN LEISHMANIASIS

2.1.1. American Cutaneous Leishmaniasis (ACL)

The first reports of ACL are found in the ancient pre-colombian potteries (Mochica, 100-600 A.D.) from Peru and Ecuador, which reproduce cutaneous and mucocutaneous lesions characteristic of this disease. There are also some reports from Spanish chroniclers that mention ulcerative lesions in the faces of Peruvian natives. Other evidence that confirmed the age of South-American leishmaniasis was the discovery of natural hosts like the armadillo and the sloths.

The relationship between ACL and the sandfly vector has already been known since 1764. At that time, the chronicler Cosme Bueno published “El Conocimiento de los Tiempos”, in which he mentioned facial ulcers that were difficult to cure and that were caused by an insect called “uta” (Herrer 1962).

From the various clinical and epidemiological studies, it is well established that the ACL extends from Mexico in the North to Argentina in the South, and is mainly located in the rain forest.
Among the main Leishmania complex that caused ACL are *L. (L.) mexicana* complex and *L. (V.) braziliensis* complex.

2.1.2. American Visceral Leishmaniasis (AVL)

Leishmania *chagasi*, a member of the *L. donovani* complex, is considered the aetiological agent of VL in America; however, the question about the possible origin of *L. chagasi* remains: is it a native or an imported species from the Old World? According to Mauricio et al. (2000) *L. chagasi* and *L. infantum* are the same species, as Cunha himself recognized in 1938 (Cunha 1938a and 1938b), based on genetic and enzymatic data which confirm the hypothesis that *L. infantum* was introduced during the colonization of America by the Europeans, probably via infected dogs.

2.2. LEISHMANIASIS IN PERU

2.2.1. Description of Peru

Peru is located on the west side of the South America continent, facing the Pacific Ocean. Geographically, Peru is located between 0° 0’ 48’’ and 18° 21’ 03’’ latitude South and 68° 39’ 27’’ to 81° 19’ 34.5’’ longitude West, representing the extreme limits of the Peruvian territory. The country borders with Ecuador, Colombia, Brazil, Bolívia and Chile. The Peruvian territory, including its islands and the corresponding portion of the Titicaca Lake, is 1,285,216 Km^2 (IGN 1989).
The Peruvian territory is dominated by the presence of the Andean Cordillera, which influences all facets of the country and the variety of landscapes and climates, plants, animals and human life.

Geography
In line with the general characteristics of the Peruvian territory, determined by the Cordillera de los Andes, Peru was traditionally divided into three main regions. The Coast is constituted by the low desert areas between the Pacific and the Andean Cordillera. The Sierra is occupied by the highlands. The Amazonian jungle (Selva o montaña) is the forested area on the east side of the Cordillera. Pulgar (1987) divided the territory expressing the gradient of different situations into eight regions, based on altitude and the predominant flora and fauna. From West to East, these regions are: Coast (0–500 m altitude), Yunga (500–2300m), Quechua (2300–3500m), Suni (3500–4100m), Puna (4100–4800m), Janca (over 4800m, snow cap), Rupa-rupa (1000–400m) or “upper jungle” and Omagua or “lower jungle” (400–0m).

Climate

The presence of the Andean Cordillera affects the climate of the whole country. The Pacific's cold water evaporates from the sea as condensed fog. When these clouds move to the continent, they become stratified by the higher temperature on the coast. The Andean highlands attract the clouds and humidity, which, on reaching the top of the mountains, provide the water that will form the summits ice cover (Moseley 1975; Mejía 1987). The Coast is a long narrow desert between the Andean Cordillera and the Pacific Ocean, where it seldom rains. The Coast strip depends on the rivers for water supply for the cities and agriculture. Most of the water from the melting ice tops from the mountains and rain of the highlands flows to the East side of the Cordillera in rivers.

The highlands – and to a lesser extent the Amazon basin, where it rains less frequently – are characterized by two well-defined seasons: a "rainy season" from January to March with heavy rains and clouds, and a "dry season" from April to December, with no rain and a dry slopes. This is in stark contrast with the climate on the Coast, where more or less gradual changes can be observed between four seasons: summer from January to March, followed by autumn, then winter from June to August, when it is constantly cloudy and almost without rain, and spring from September to December.

Most Old World leishmaniasis occurs in open semi-arid or even desert conditions. On the other hand, New World CL is still mostly associated with forest below sea level (e.g., the Peruvian tropical rainforest) or high altitudes (Peruvian Andes), and MCL is present mainly in the Amazonian jungle. CL foci have a wide ecological variation (like the one found in the greater part of the Peruvian territory) and sandflies are able to find cool, shaded, humid microhabitats.

2.2.2. History

In the past, the population of Peru, located in the Pacific coast of South America, was mainly concentrated in the coast and highlands, while the forest areas on the East side of the Andes were sparsely inhabited (Kauffman 1980). The various pre-Inca and Inca cultures that developed in these areas were exposed to and affected by several diseases throughout their history. In the case of leishmaniasis, they were familiar with and recognized the disease, as we have been able to corroborate by the very well conserved ceramic pottery they made to depict the lesions they observed. The early inhabitants of the current Peruvian territory made pottery vessels for use in daily life, and frequently decorated them with human, animal or vegetable designs. These pots were also used in burials for food, water and ceremonial rituals for the deceased, many of which have been found in pyramidal burial constructions called "huacas", from which the name of the vessels, "huacos", is derived.

It is very likely that some of the images on these "huacos" refer to leishmaniasis (Fig. 5). Their age, usually over 2000 years, indicates that this disease transmitted by *Lutzomyia spp*. already existed in Peru before the Spanish conquerors came to America. It was Jimenez de la Espada who first suggested this hypothesis in 1897, in a discussion of the images on Peruvian pottery (Lehmann-Nitsche 1899).

Furthermore, Urteaga (1991) found a Peruvian Inca mummy skull showing reduction of the nasal septum with rounded borders, which suggested that this was a case of MCL. The long existence of leishmaniasis is also evidenced by the understanding and knowledge the local population have about it, including aspects of its general epidemiology, clinical development, and treatment with traditional medicines.

There are also some Spanish chronicles, explorers and missionaries that recorded early knowledge of the causes of this disease. In 1586, Fray Rodrigo de Loáis was the first to describe leishmaniasis in Peru in some of his manuscripts. The various popular beliefs were usually associated with the environment in which the affected people lived. Insects were commonly thought to be the cause of the disease, and even sometimes corresponded with the description of a sandfly (Lumbreras & Guerra 1985).

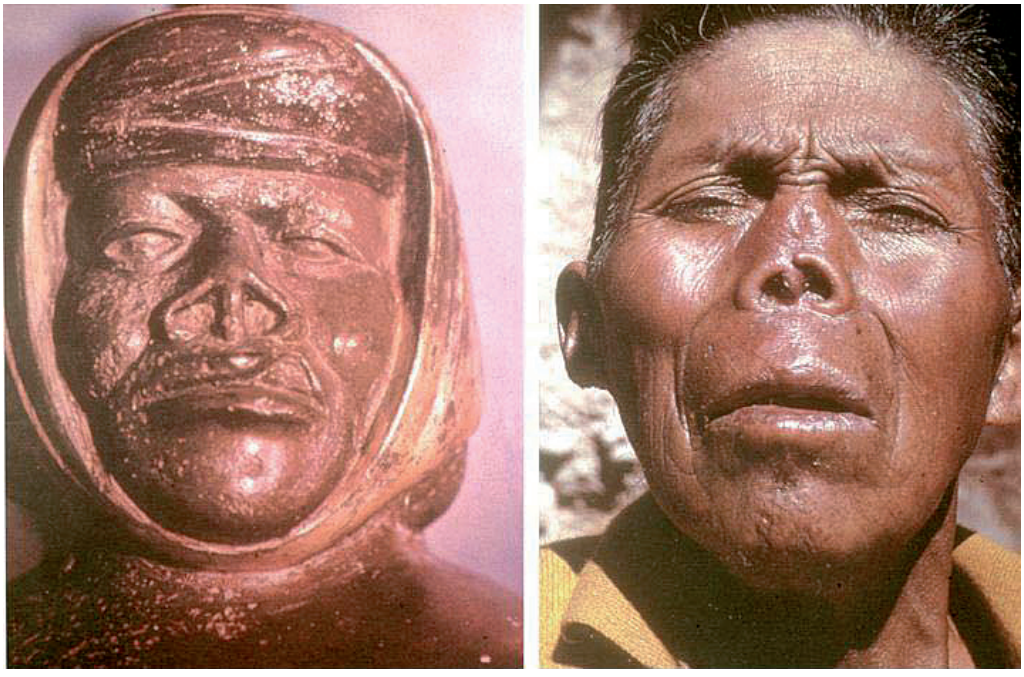

Fig. 5 A *pre-Inca pottery compared with a Bolivian case of leishmaniasis (http://www.who.int/-leishmaniasis/en/)*

2.2.3. Current situation

2.2.3.1. Clinical forms in Peru

***Andean* leishmaniasis:** this cutaneous form "Uta" is produced by *L. (V.) peruviana* and is mainly found between 600 to 3000m above sea level (a. s. l.) on the western slopes of the Andean and inter-Andean valleys. Uta and Bartonellosis share the same endemic areas mainly due to the distribution of the sandfly vector, which is the vector for both diseases. The proximity of reservoirs, vector and human dwellings resulting from the geography and local human activities make the transmission of Andean leishmaniasis to humans very efficient. The Uta lesions are localized ulcers with lymphatic involvement, which some-

times appear as multiple lesions, popular or nodular, ulcerated or not, and usually painless and last between 8 to 10 months (Lumbreras and Guerra 1985).

***Amazonian* leishmaniasis:** the Mucocutaneous form "Espundia", essentially caused by *L. (V.) braziliensis,* can be found on the eastern slopes of the Andes beginning from 800 m a. s. l. down to sea level in the low jungle. The transmission of Espundia is mainly accidental and only occurs in selected groups of people (e.g., fisherman, hunters, gold seekers, and wood workers) who are active in areas inhabited only by animals and vectors. The ulcerative lesions in the Espundia are more severe and its progression is, apparently, more rapid than in Uta. Metastatic lesions appear in the mucosal tissues in 10% of the cases and produce extensive disfiguration and pain (Lumbreras and Guerra 1985). In addition, lesions due to *L. (V.) braziliensis* are also more difficult to treat. A recent study has shown that the risk of treatment failure with antimonials was 22 times higher in patients infected with *L. (V.) braziliensis* than in patients infected with *L. (V.) guyanensis*, and 10 times for *L. (V.) peruviana* (Arevalo et al. 2007)

2.2.3.2. Leishmania and sandflies species reported in Peru

Five species of the *Leishmania* genus have been reported as causing human disease in Peru (Perez 1995). The three main species are labelled with an asterisk below (Fig. 6):

- Leishmania *(Leishmania) amazonensis;* Lainson & Shaw, 1972
- Leishmania (Viannia) braziliensis; Vianna, 1911, emend. Matta, 1916*
- Leishmania (Viannia) guyanensis; Floch, 1954*
- Leishmania *(Viannia) lainsoni;* Silveira, Shaw, Braga & Ishikawa, 1987
- Leishmania (Viannia) peruviana; Vélez, 1913*

Fig. 6 *Geographic distribution of Leishmania (Viannia) braziliensis, L. (V.) peruviana and L. (V.) guyanensis by department in Peru (Arevalo et al. 2007).*

In addition, *L. (V.) braziliensis/L. (V.) peruviana* phenotypic hybrids have been reported (Dujardin et al. 1995a, Nolder et al. 2007)

In Peru, leishmaniasis is transmitted by sandflies of the genus *Lutzomyia* (Villaseca 1993; Caceres 2004), which includes 137 species. The diversity is highly variable: in the Andean and inter-andean valleys where *L. (V.) peruviana* can be found, there are between 4–8 *Lutzomyia* species (4 species in the Rimac river valley, Lima), while in the Amazon region where *L. (V.) braziliensis* is predominant, there are between 30–42 species (38 species in Tambopata, Madre de Dios). From the 137 species, only 4 have been found to be Leishmania vectors in Peru, *L. ayacuchensis*, *L. peruensis*, *L. tejadai* y *L. verrucarum* (Perez 1995).

Classification of the Genus *Lutzomyia*

Class:	Insecta
Order:	Diptera
Suborder:	Nematocera
Family:	Psychodidae
Subfamily:	Phlebotominae
Genus:	*Lutzomyia*

2.2.3.3. Epidemiology of Leishmaniasis in Peru

Leishmaniasis cases have been reported from a total of 557 Peruvian localities (58.49% of the national territory), with a population of around 8 millions inhabitants (28% of the total population). Up to October 2007, 7193 confirmed and 1490 possible cases of CL and 279 confirmed and 75 possible cases of ML were reported. The total incidence is 31.47 and 1.28 (per 100,000 habitants) for Cutaneous and Mucocutaneous leishmaniasis respectively (DGE-MINSA 2007).

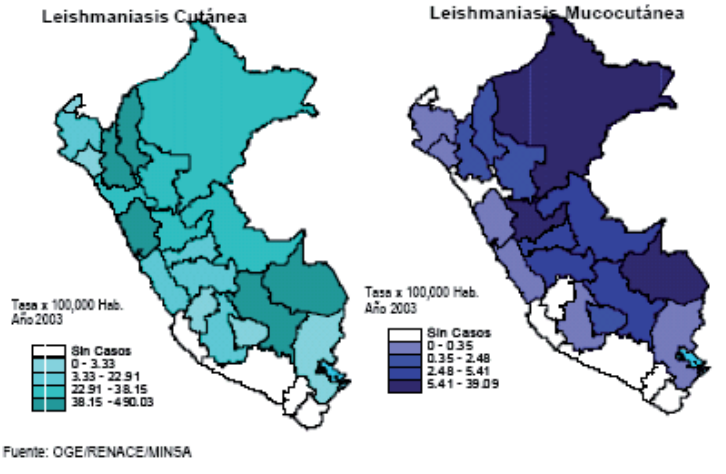

Fig. 7 *Last map reported by the Peruvian Minister of Health about the Index case in Cutaneous and Mucocutaneos leishmaniasis respectively (OGE-MINSA 2003).*

3. PATHOLOGY, PATHOGENECITY AND VIRULENCE

Pathology is the outcome of a host-parasite relationship in the broadest sense; the host-parasite interaction within the macrophage is detailed in the next section.
CL is characterized by the colonization of the mononuclear system cells by amastigotes producing the granulome as a result of the aggregation of infected macrophages, lymphocytes, other immune cells and plasma cell infiltration (Ridley 1987). Other characteristics relating to CL lesions include disorganization of connective dermis tissues, hyperplasia of the epidermis and, in some cases, blood vessel involvement. There is a predominance of Th1 response, exacerbating cell immunity and few circulating antibodies (serology is not useful for diagnosis).
In the case of MCL, the unique characteristic is the late development of metastatic lesions to the oronasal mucosa producing mutilating lesions ("espundia"). The mucosal lesions are located mainly in the deep mucosa where edema and congestion occur, with a heavy plasma cell infiltrate and a unique proliferation of the vascular endothelial cells.
DCL occurs in about 1 per 10,000 infections with *L. aethiopica* (Bryceson 1969). *L.* amazonensis infection also results in this form. In both cases the diffusion is associated with Leishmanial antigen-specific defective cell-mediated immune response. The primary lesion does not ulcerate but the infection spreads slowly through the bloodstream and relocates elsewhere. The clinical picture often resembles lepromatous leprosy and causes grotesque deformity. Spontaneous healing is rare in this case (Bryceson 1969).
There are more common features than differences in the pathology of VL (kala-azar) in all the endemic areas. VL is a systemic disease of the mononuclear phagocyte system. There is predominance of Th2 response, depressed cell immunity, abundance of antibodies (serology being a useful method for diagnosis). In advanced cases, almost all the body parts are involved and the parasite load can be enormous. The main organs affected are the liver and spleen, but the bone marrow, lymph nodes and skin are also commonly involved. The infected cells are both diffuse and aggregated in granulomata, and associated with some cellular infiltrate in which plasma cells usually predominate. The infiltrate is, however, relatively less extensive than in CL. The causes of dead, as consequence of VL, are heart failure, pericarditis, bleeding, anaemia, uraemia, sepsis, bronchopneumonia, dysentery, toxemia and wasting (Ridley 1987). VL is an important opportunistic infection in persons with suppressed cell-mediated immunity (Medrano et al. 1992) and a number of cases have been reported after organ transplantation (Basset et al. 2005).

(Muco-) cutaneous leishmaniasis, like any other clinical form of the disease, has most probably a multi-factorial origin. Pathogenesis is explained by an exacerbated cellular response (Bacellar et al. 2002), which might be related to the host's immunity and genetic background, parasite factors or even immuno-modulatory effectors present in the sand-fly's saliva.

3.1. HUMAN HOST: GENETIC AND IMMUNOLOGICAL ASPECTS

Early reports highlighted the contribution of human genetic components in controlling susceptibility to clinical leishmaniasis and influencing severity of and resistance to the disease among healthy individuals. This was shown by comparing ethnic groups (Bolivia: MCL due to *L. (V.) braziliensis*, Walton et al. 1977), natives and migrants (Bolivia: CL and MCL due to *L. (V.) braziliensis*, Alcais et al. 1997ab) or by familial clustering studies (Peru: CL due *L. (V.) peruviana*, Shaw et al. 1995). The latter study favoured a multifactorial over a sporadic model (Shaw et al. 1995). Identification of the candidate genes or regions involved in the genetic control of leishmaniasis was made possible by a 'mouse-to-human' strategy, refined by the knowledge of the human immune response to leishmaniasis (e.g., the Th1/Th2 balance) and genetic studies of other intra-macrophage pathogens, like mycobacteria (Blackwell 1998). However, all the mouse studies used the *L. (L.) major* model (Hadelkova et al. 2006; Foote and Handman 2005; Roberts et al. 1997; Lipoldova et al. 2002; Fowell and Locksley 1999; Elso et al. 2004; Kropf et al. 2004; Wei et al. 2004) and – considering the diversity in the parasite approach of the host mentioned above – it is unknown whether host genetic determinants of leishmaniasis will be the same for different Leishmania species. In addition, so far there has been a strong unbalance between the number of experimental analyses in mice and studies in a natural human context. The few reports on humans yet available indicate a role of HLA molecules in MCL (Brazil: *L. (V.) braziliensis,* Petzl-Erler et al. 1991) and CL (French Guiana: *L. (V.) guyanensis*, Barbier et al. 1987; Venezuela: *L. (V.) guyanensis* and *L. (V.) braziliensis*, Lara et al. 1991; Mexico: *L. (L.) mexicana*, Olivo-Diaz et al. 2004) and TNF in MCL (Venezuela: *L. (V.) braziliensis*, Cabrera et al. 1995).

With respect to the balance between the T Helper cell response, Th1 (which protects against intracellular pathogens) and Th2 (linked to allergy or protection against extracellular pathogens), three hypotheses have been proposed in experimental leishmaniasis (Reiner and Locksley 1995): (i) different peptides stimulate distinct groups of Th1 or Th2 clones, (ii) a particular pattern of cytokines and cofactors, produced by innate immune system accessory cells, would be the reason for the divergence, (iii) under stimulation, T cells of different mice strains would have an innate tendency for the development of one of the two poles of the response (clones of T cells derived from C57BL/6 mice, for example, would take the Th1 direction while cells from BALB/c would go for the Th2 pole).

3.2. PARASITE: EFFECTS AND FACTORS

In order to be a successful invading organism, Leishmania parasites need virulence factors allowing the parasites to disrupt the host's immune barrier and avoid or use the elicted immune response to their own benefit. There are three main categories of these virulence factors: (i) invasive or evasive determinants (lipophospoglycans, leishmanolysin, or cysteine proteases), which are crucial to infection but unable to produce pathology in the host; (ii) pathoantigenic determinants (histones, chaperones, or proteasomes), which lead to host immunopathology as the principal cause of clinical symptoms; and (iii) protective

determinants (to be identified), which seem to lead to clinical cure (Chang and McGwire 2002; Chang et al. 2003).
While some studies indicate a correlation between specific parasite genotypes and clinical forms (Guerbouj et al. 2001), others (Schonian et al. 2000) did not find this correlation. Nevertheless, it is a fact that the parasite's genetic diversity is its major advantage, and it seems that Leishmania *spp*. differ in their approach to tackling the host immune system (Reithinger et al. 2007). It is recommended to look for other molecular markers for genotyping natural populations and focus on the polymorphism of virulence determinants (Dujardin et al. 2002). For instance, in *L. (V.) peruviana*, a strain with high pathogenicity but low virulence, Victoir et al. (1995) found a deletion of half of the leishmanolysin genes compared to the *L. (V.) braziliensis* strain, with low pahogenicity but high virulence. This kind of approach should be complemented by studies at transcriptomic and proteomic levels (Reithinger et al. 2007).
There are other factors (conditions) that allow the parasite to infect host cells. It is well documented that metacyclic forms of Leishmania parasites are more resistant to complement lyses than are log phase (procyclic promastigotes) and, as a consequence, are more infective in *in vitro* and *in vivo* models (Franke et al. 1985; Sacks & Perkins 1985; Sacks 1989; Gamboa et al. 2007). Puentes et al. (1988) also suggested that metacyclics interaction with serum complement could be different from procyclic promastigotes (activation of classical and alternative pathway respectively). In 2002, Dominguez et al. confirmed previous data from Mosser et al. (1986), establishing that both complement pathways are activated.

3.3. MACROPHAGE: ITS INTERACTION WITH LEISHMANIA

When confronting an intracellular pathogen, the macrophages can have two roles: they can be important effector cells that control and eliminate the invading organism through oxidative and non-oxidative mechanisms, or they can act as long-term host cells and facilitate the multiplication and survival of the pathogens, protecting them from the toxic effects of the extracellular medium.
In the case of Leishmania, the parasites not only found the mechanisms to avoid the immune response from the host, but also use the immune response to their own benefit. For instance, the complement proteins (system) of the serum allow the parasites to interact with the macrophage surface. There are also other molecules – such as the glycoprotein receptors (lectin) on the macrophage surface – that interact with glycoproteins or glycolipids on the parasite surface (Blackwell & Alexander 1983). Other evading mechanisms that allow the survival of the Leishmania parasite are reviewed by Bogdan and Rollinghoff (1999). These include a positive or negative modulation in the production of Nitric Oxide (NO) depending on the stimulating conditions: a down-regulation in the production of reactive oxygen radicals through the NADPH oxidase system, apoptotic modulation in order to prolong the viability of the macrophages to assure the parasite dispersion, modulation at the level of cytokines production, and others.
The first step in the macrophage infection by the Leishmania parasite is its internalization by simple phagocytosis forming the "Phagosome" structure, which suffers additional

modifications when it interacts with secondary lysosomes and forms the phagolysosome organelle, where the parasites are well protected from the toxic effects of the acidic pH and the lysosomal enzymes; there is also a mechanism called "circular phagocytosis", in which the complement receptors CR1 and CR2 might play an important role (Blackwell and Alexander 1983). In this new environment, besides the acidic pH, the parasites also find an increased temperature (37°C) that forces them to transform into amastigotes; this implies not only morphological changes (round-shape, without flagellum), but changes at the level of gene expression as well (Handman 1999).

3.4. VECTOR: SALIVA EFFECT

The role of the saliva from the sandfly vector is another issue that should be taken into account for the understanding the pathogenicity of Leishmania. It helps not only with the vessel localization and dilatation, but also avoids the blood coagulation and allows the parasite survival for the establishment of the initial infection in the mammal host (Handman 1999). During vector-host interaction there are some mechanisms activated from both sides. At the host level, the natural defense mechanisms will be activated due to the presence of components from the complement system, thrombin (coagulation), kinins (vasoconstriction), platelets, natural antibodies, phagocytes, etc, at the site of inoculation (Gillespie et al. 2000; de Almeida et al. 2003). At the level of the vector, the pharmacological agents of the saliva are maxadilan and adenosine (vasodilatatory agents found in *Lutzomia* and *Phlebotomus* respectively), interleukin 2 (IL-2) binding factor, apyrase (anti-platetelet aggregation agent found in *Lutzomia* and *Phlebotomus*), prostaglandin E2 (vasodilatatory and immunossupressive actions), etc. (Ribeiro 1987; Gillespie et al. 2000).

4. STRUCTURE, DYNAMICS AND EXPRESSION OF THE GENOME

4.1. THE GENOME

There are two types of DNA in the Leishmania genome: nuclear DNA and mitochondrial DNA (kinetoplast DNA). The genome of Leishmania has an estimated size of 3.3–6.5x10e7 base pairs (or 0.116 pg of DNA/cell).

Even though the number of chromosomes is variable, the organization and gene order remains the same between the species (Tamar et al. 2000). In general, the haploid genome of Leishmania includes 36 chromosomes (Wincker et al. 1996), with the exception of *L. (V.) braziliensis* and *L. (L.) mexicana,* which have 35 (short sequence exchange between chromosomes 26 and 35, and a fusion of chromosomes 20 and 34) and 34 (two chromosomal fusions: 8+29 and 20+36) chromosomes respectively (Britto et al. 1998).

The Leishmania genome is rich in GC (64%) and it encodes for approximately 8300 genes. The 36 chromosomes of the 32.8 megabase haploid genome of *L. major* (Friedlin strain) was sequenced by the Sanger Institute (www.GeneDB.org), which predicts 911 RNA genes, 39 pseudogenes and 8272 protein-coding genes, including genes involved in host-pathogen interactions, such as proteolytic enzymes, an extensive machinery for synthesis of complex surface glycoconjugates (Ivens et al. 2005).

Comparing the genome of *L. (L.) infantum* and *L. (V.) braziliensis* with the published sequence of *L. (L.) major* (from the 8395 annotated genes, 8195 and 8314 were found in *L. (L.) infantum* and *L. (V.) braziliensis* respectively), Peacock et al. (2007) found marked conservation synteny and identified only ~200 differences at the gene or pseudogene content level with 78 genes that are restricted to individual species. There is also evidence of the existence of RNA-mediated interference (RNAi) machinery and transposable elements in the genome of the most divergent species, *L. (V.) braziliensis* (Peacock et al. 2007). Further analysis showed that *L. (V.) braziliensis* possess 47 genes that are absent in the other two species, *L. (L.) infanum* has 27 specific genes and *L. (L.) major* has only five (Smith et al. 2007) (Fig. 8).

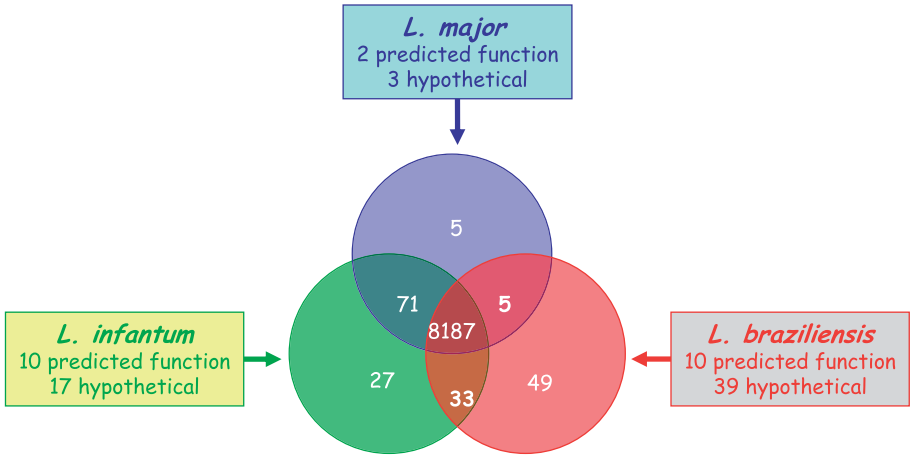

Fig. 8 *Leishmania species-specific genes. The three species share 8187 protein-coding genes. The species-specific genes are subdivided into those with a predicted function and those with an unknown function at the time of publication (Smith et al. 2007).*

4.2. GENOMIC ORGANIZATION AND DYNAMICS

Analysis of the Leishmania genome organization has shown three different components of nuclear DNA: a highly repetitive component occupying 12% of the genome; a middle repetitive component occupying an additional 13% of the genome; and unique sequences constituting 62% of the genome (Leon et al. 1978). The repetitive sequences of Leishmania are usually not chromosome-specific and are not necessarily shared by all chromosomes (Bastien et al. 1992). Microsatellite DNA (di- tri- and tetra-nucleotide repeat motifs) is present on the chromosome of several *Lesihmania spp*., where (CA)n repeats are the most common (Rossi et al. 1994).

The size of each chromosome is variable. Up to 25% of the chromosomal length could be observed, probably due to events of amplification/deletion. The chromosomal karyotypes have been observed to be unstable during *in vitro* culture (Dujardin et al. 2007), and a significant level of polymorphism have been found at an interspecies and intraspecies level in PFGE-based studies (Bastien et al. 1992; Dujardin et al. 1995b). The genetic basis

for this polymorphism is mostly related to mechanisms of DNA amplification/deletion and involves essentially tandemly repeated genes (Pages et al. 1989). This has been shown for mini-exon genes on *L. (L.) major* chromosome 2 (Iovannisci and Beverley 1989), in the size of chromosomes bearing rDNA and mini-exon genes correlated with eco-geography in Leishmania subgenus *Viannia* (Inga et al. 1998; Kebede et al. 1999), or in chromosomes bearing gp63, PSA2, K39 mini-exon and rDNA genes in *L. (L.) infantum* (Guerbouj et al. 2001).

4.3. GENE EXPRESSION AND REGULATION

Leishmania genes are polycistronically transcribed, and two mechanisms allow the generation of monocistronic RNAs from the precursor RNAs: (i) the 5' mini-exon addition by trans-splicing mechanism: 39-nucleotide Spliced Leader (SL) RNA, also known as mini-exon, addition to the 5' end of all pre-mRNA, it is donated by a precursor SL RNA encoded separately in the genome (Stiles et al. 1999); and (ii) the 3' polyadenylation process: RNA processing reaction which involves cleavage at the polyadenylation site with the concomitant addition of the poly (A) tail to the 3' end of the newly formed mRNA (Nilsen 1995, Ullu and Nilsen 1995). The degree of polyadenylation is variable between different strains and different life stages (promastigotes and amastigotes) (Decuypere et al. 2005).

So far, introns (characteristic of genomic organization in higher eukaryotes) have not been discovered within any of the Leishmania protein-coding genes (Myler et al. 2000), although cis-splicing mechanisms have recently been discovered in other trypanosomatids (Mair et al. 2000).

The consensus poly-(A) signal sequences identified in higher eukaryotes are not present in the 3' UTR of Leishmania genes. The coupling of trans-splicing and polyadenylation reactions in trypanosomatids transcripts may substitute for poly-(A) signals in Leishmania (Stiles et al. 1999).

The regulation of the expression of Leishmania genes is not well understood. There is no regulation at transcription level described for Leishmania (Clayton 2002); the only promotors described for Leishmania relate to ribosomal genes (Yan et al. 1999) and the spliced leader RNA genes (Yu. et al. 1998). The gene expression in Leishmania is controlled at post-transcriptional level, probably during the trans-splicing and polyadelylation steps and also mediated by 3' untranslated regions (UTR) and intergenic sequences (McCoy et al. 1998; Boucher et al. 2002; Rochette et al. 2005; Wu et al. 2000), making this system more rapid and efficient. It is suggested that labile protein factors could affect the degradation/stability of precursor of mature transcripts (Graham and Barry 1995; Stiles et al. 1999). In addition, RNA interference has been mentioned as a way of regulating gene expression, replacing (in part) promoter driven gene regulation. Rusconi et al, BMC Biotech 5, 6 (2005), Uller et al, Cell Microbiol 6, 509 (2004), Smith et al, (2007).

5. THIS THESIS

5.1. PREVIOUS WORK RELATED TO THIS STUDY (BACKGROUND)

The research groups at Institute of Tropical Medicine "Prince Leopold" in Antwerp (ITG) and the Institute of Tropical Medicine "Alexander Von Humboldt" (IMTAvH-UPCH) have performed extensive studies of the *L. (V.) braziliensis/L. (V.) peruviana* model at genomic level. Although in evolutionary terms both species appeared to have diverged recently (16), important chromosomal rearrangements that affect essential genes have been found: gp63, rDNA, mini-exon and cystein proteinases. Interestingly, the less pathogen *L. (V.) peruviana* displayed a smaller size of these four chromosomes, together with a corresponding reduction in the copy number of essential genes (mini-exon (Kebede et al. 1999), cysteine proteinase b (Polet 1999), gp63 (Dujardin et al. 1994; Victoir et al. 1995) and rDNA (Inga et al. 1998)), and with deletion of a *L. (V.) braziliensis*-specific gp63 isogene (Victoir et al. 1998). Based on these data, the work hypothesis is that these important genomic rearrangements occurring in *L. (V.) braziliensis* and *L. (V.) peruviana* could be responsible for qualitative and quantitative differences in gene expression, potentially related to different phenotypes. Similar effects were reported in other micro-organisms (Malezka & Clark-Walker 1993; Ramamoorthy et al. 1995; Bourke et al. 1996).

Therefore, the studies reported in this thesis focuses on gene expression level, which can be examined by two complementary approaches, one analyzing specific genes reported to be involved in virulence, and the other randomly analyzing whole populations of transcripts.

5.1.1. Targeted studies

In a parallel PhD thesis, Victoir (2001) specifically analyzed the *gp63* genes, one of the main virulence factors of Leishmania. She described eight different gene families in *L. (V.) braziliensis* (Victoir et al. 2005). Within the same organism, these genes showed a nucleotide sequence varying in certain stretches from 3 to 34% as well as a mosaic structure. Functional implications of these results were explored from predicted *L. (V.) braziliensis* protein sequences: regions encoding the *gp63* catalytic site showed a conserved sequence, while regions encoding surface domains possibly involved in the host/parasite interaction (macrophage adhesion sites and immunodominant B-cell and T-cell epitopes) were variable. It was speculated that this was an adaptive strategy of the parasite. Using a gp63-specific RT-PCR assay, Victoir found some minor transcripts that might be specifically related to the amastigote life stage. However, a dominant picture of constitutive gp63 gene expression was reported in both *L. (V.) braziliensis* and *L. (V.) peruviana*, which might be explained by problems in the quality of the biological material used (e.g., absence of purification of metacyclic stages). Gene expression in *L. (V.) braziliensis* and *L. (V.) peruviana* was also compared to analyze the consequences of the genomic differences observed between both species. RT-PCR RFLP analysis revealed that – like in genomic analyses – the *L. (V.) braziliensis* strains studied (LC2043 and LC2177) were more homogeneous than *L. (V.) peruviana* strains (LCA08 and HB86). Indeed, a clear

difference could been observed between the gp63 expression pattern of the *L. (V.) peruviana* strain of Northern Peru and that of the strain originating from South Peru. Furthermore, the *L. (V.) braziliensis*-specific gp63 genes identified by genomic analysis were found to be transcribed throughout the parasitic cycle. Finally, an unusual association between a partial gp63 gene and a subtelomeric sequence was consistently identified at the mRNA level in both *L. (V.) braziliensis* strains, but rarely or not at all in *L. (V.) peruviana*. This association is probably a particular case of mosaic gp63 genes occuring at chromosomal extremities and witnesses the high level of intragenic recombination occurring within that locus. Its exact function (protein or regulatory element) as well as its possible role in the difference in pathology between *L. (V.) braziliensis* and *L. (V.) peruviana* remain to be explored.

5.1.2. Random studies

The targeted approach has the advantage of directly focusing on candidate genes found to be candidate virulence factors in other species. However, virulence factors might be species-related, and this approach might also neglect interesting new factors. In addition, in the case of gp63, the genetic structure of that locus appeared to be very complex (eight different gene families and difficulty to design specific RT-PCR assays for each of them). Therefore, a complementary approach, 'fishing' new potential virulence markers by a global analysis of mRNA populations, can be applied. Several PCR-based methods have been developed for studies of complex populations of RNAs without knowledge of sequence information (e.g., differential display, Liang et al. 1992; subtractive hybridization, Diatchenko et al. 1996 and microarrays, Schena et al. 1995).

Differential Display analysis (DD) (Liang et al. 1992) provides a cDNA fingerprint of particular cells and allows the subsequent isolation and further characterization of cDNAs that are potentially of interest. DD analysis was recently used to identify genes preferentially expressed in amastigotes of. *L. (L.) major* (Ouakad et al. 2007).

In suppression subtractive hybridization (SSH), cDNAs of two strains with different phenotypes (and thus different transcript populations) are used to make a subtraction library, likely enriched in cDNA specific to one of the two strains. After a series of screening steps in order eliminate false positive, the clones can be sequenced. This approach was already applied in Leishmania to search for drug-resistance markers, but was hampered by the presence of poly-adenylated rRNA (Decuypere et al. 2005).

Microarray analysis were performed to study gene expression profiles in order to obtain new information about the dynamics of transcript abundance during Leishmania development (procyclics, metacyclics and amastigotes stages) using *L. (L.) major Friedlin* and LV39 strain (Saxena et al. 2003 and Almeida et al. 2004, respectively). Also, microarrays from *L. (L.) major* were applied for related Leishmania strains like *L. (L.) mexicana* (Holzer et al. 2006). In order to have complete information for gene expression in different strains or isolates, microarrays have to be developed specifically for each Leishmania strain. These can then be tested at different life stages and under different conditions (stress due to temperature, pH, treatment, clinical outcome, etc.). This kind of technique is

quite expensive and can only be used as a first screening, which means that alternative techniques need to be developed for developing countries.

5.2. AIMS AND OUTLINE OF THIS THESIS

The two neotropical species *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana* are found in different environments and are associated with two very different clinical forms, *L. (V.) braziliensis* being far more pathogenic in humans than *L. (V.) peruviana*. Despite this difference, the two parasites are genetically very similar. This clinical pleomorphism could be due to environmental, host and/or parasite factors.

The general objective of this thesis was to explore the contribution of parasite factors to the development of the pathology by analyzing genomic make-up and stage characteristics.

In the first experimental chapter (chapter 2), we aimed to contextualize the evolutionary history of both species, using a rapidly evolving genetic marker. Therefore, the chromosomal size of different strains originating from different regions of Peru was processed with a specific algorithm and used to develop an evolutionary hypothesis.
We hypothesize that *L. (V.) peruviana* descends from *L. (V.) braziliensis* and acquired its 'peruviana' character during the southward colonization and adaptation of the transmission cycle in the Peruvian Andes.

Chapter 3 describes how a combination of classic techniques (collection and dissection) can be combined with state of the art molecular techniques such as PCR to detect and identify Leishmania parasites in sandflies.

In chapter 4, we aimed to verify whether both species showed phenotypic differences in terms of virulence in experimental models. Therefore, an *in vitro* and *in vivo* model were developed and validated on four reference strains, two representative of *L. (V.) braziliensis* from cutaneous and mucosal origin and two representative of *L. (V.) peruviana* from Northern and Southern Peru respectively.
Our models were sufficiently reproducible and sensitive to detect phenotypic differences among strains. We showed *in vitro* as well as *in vivo* that *L. (V.) braziliensis* was more infective than *L. (V.) peruviana*. We also found that the *in vitro* infectivity patterns were in line with the geographical gradient of parasite populations evidenced previously.

In a fourth experimental chapter (chapter 5), we aimed to identify transcripts that might be (i) candidate markers of the infective stages of *L. (V.) braziliensis* (metacyclics and intracellular amastigotes) and (ii) potential controls, i.e. constitutively expressed; these markers might serve as reference for future gene expression profiling studies in *L. (V.) braziliensis* and *L. (V.) peruviana.* Therefore, we applied differential display to screen transcripts originating from different biological stages of a reference *L. (V.) braziliensis* strain.

We found two genes that were more expressed in metacyclics (infective insect stage-specific protein, *'meta1'*) and amastigotes (oligopeptidase b, *'opb'*) respectively. In addition, two genes (glucose-6-phosphate dehydrogenase and serine/threonine kinase, *'g6p'* and *'stpk'*, respectively) were shown to be similarly expressed in the different infective stages.

In a fifth experimental chapter (chapter 6), we aimed to monitor the expression of the four genes, identified as described in Chapter 3, in *L. (V.) braziliensis* and *L. (V.) peruviana*. Therefore, we used the four reference strains described in Chapter 2, and followed the expression of the four genes during the *in vitro* life cycle by real-time quantitative PCR.
We found, in the case of *'meta1'* gene, an increased relative expression level at stationary ("infective") stage (days 4 and 5) in the four reference strains. The *'opb'* was found to be over-expressed in amastigote stages of *L. (V.) braziliensis,* while no signal was detected in *L. (V.) peruviana.* Interestingly, *'opb'* gene expression level was found to be higher in the *L. (V.) braziliensis* strain of mucosal origin than in the cutaneous strain.

CHAPTER 2

Genomic Rearrangements in Trypanosomatids: an Alternative to the "One Gene" Evolutionary Hypotheses?

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Most molecular trees of trypanosomatids are based on point mutations within DNA sequences. In contrast, there are very few evolutionary studies considering DNA (re) arrangement as genetic characters. Waiting for the completion of the various parasite genome projects, first information may already be obtained from chromosome size-polymorphism, using the appropriate algorithms for data processing. Three illustrative models are presented here. First, the case of Leishmania (Viannia) braziliensis/L. (V.) peruviana *is described. Thanks to a fast evolution rate (due essentially to amplification/deletion of tandemly repeated genes), molecular karyotyping seems particularly appropriate for studying recent evolutionary divergence, including eco-geographical diversification. Secondly, karyotype evolution is considered at the level of whole genus* Leishmania*. Despite the fast chromosome evolution rate, there is qualitative congruence with MLEE- and RAPD-based evolutionary hypotheses. Significant differences may be observed between major lineages, likely corresponding to major and less frequent rearrangements (fusion/fission, translocation). Thirdly, comparison is made with* Trypanosoma cruzi. *Again congruence is observed with other hypotheses and major lineages are delineated by significant chromosome rearrangements. The level of karyotype polymorphism within that "species" is similar to the one observed in "genus"* Leishmania*. The relativity of the species concept among these two groups of parasites is discussed.*

Key words: *Leishmania - Trypanosoma cruzi* - chromosome evolution

The DNA sequence and its variation – essentially by point mutations – underlie the discipline of molecular evolution. Methods of analysis and evolutionary models are robust and, according to the grade of conservation of the gene or intergenic region considered, studies at different evolutionary scales can be performed. Nevertheless, the adequacy between "one gene" trees, and "species" trees might be questioned. Indeed, only orthologous genes (homology resulting from speciation; Wiley 1981) should be used and several genes should be sequenced to confirm orthology and discard paralogous (homology resulting from gene duplication) and xenologous (result of gene transfer) genes (Larson 1994, MacIntyre 1994). An other advantage of multiple gene analysis is the possibility of exploring different metabolic pathways and thus getting a more global insight on evolution. This concept has been extensively – albeit indirectly – explored by multi-locus enzyme Electrophoresis (MLEE, Bañuls et al. 1999).

However, according to Danchin (1998), genomes are not merely collections of genes, and the map of the cell would be in the chromosome (Danchin & Hénaut 1997). In other words, rearrangement of genes can either induce or reflect evolutionary changes. This hypothesis proposed for prokaryotes (Danchin & Hénaut 1997) is documented in higher eukaryotes too and, according to Wilson et al. (1974), gene rearrangements would be more important than point mutations as sources for evolutionary changes. However, very few evolutionary studies have considered gene arrangement as molecular character in trypanosomatids and other

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protozoa, despite their primordial phylogenetic position between prokaryotes and eukaryotes.

MECHANISMS OF GENE REARRANGEMENT AND APPROACHES FOR THEIR ANALYSIS

A first, direct approach to gene rearrangements can be considered, in which the organisation of specific genes is compared in different taxa. This concept has been applied to the study of a large genomic region containing glucose transporter genes in *Trypanosoma brucei, T. congolense*, *T. vivax*, *T. cruzi* and *Leishmania donovani* (Bringaud et al. 1998). Gene organisation was shown to be identical in the three Salivarian trypanosomes, but in *T. cruzi* and *Leishmania*, insertion of additional genes was observed. Phylogenetic reconstruction based on glucose transporters was in agreement with the monophyly of genus *Trypanosoma* and the early separation of *T. vivax* from the other Salivarian trypanosomes. Another example concerns the α- and β-tubulin genes which are linked and organised in alternated tandem repeats in *T. cruzi* (Maingon et al. 1988, Cano et al. 1995) and *T. brucei* (Tomashow et al. 1983), while they are unlinked in *Leishmania* and *Sauroleishmania* (Dujardin 1995, Wincker et al. 1996, Britto et al. 1998). Furthermore, within *Leishmania*, three types of β-tubulin gene organisation have been encountered, with gene copies on (i) 3 chromosomes (8/29, 21 and 32 in New World subgenus *Leishmania*, or NWL and, 8, 21 and 32 in Old World subgenus *Leishmania*, or OWL; Wincker et al. 1996, Britto et al. 1998), and (ii) 4 chromosomes (1, 8, 21, and 32 in subgenus *Viannia*, or NWV; Dujardin 1995, Britto et al. 1998). As globins in mammals (Dover et al. 1982, Jeffreys 1982), it is likely that tubulin genes arose from a single copy gene that duplicated, diverged towards α- and β-tubulin genes, and then began to spread differentially among the genome of the different trypanosomatids. The tracking of this spread among key trypanosomatid taxa would pave the way to new evolutionary hypotheses. However, as for DNA sequence analysis, "one gene rearrangement" trees might also be questioned, and rearrangement of different genes should be studied. This should be feasible in a near future through completion of the different projects of parasite genome sequencing.

A second, indirect approach to gene rearrangement is provided by molecular karyotyping and analysis of chromosome-size variability. This approach infers that chromosome size-polymorphism is reflecting gene rearrangements and it allows to explore the whole nuclear genome. Within genus *Leishmania* and within *T. cruzi*, there is conservation of most linkage groups (Henriksson et al. 1995, Wincker et al. 1996, Britto et al. 1998), and chromosome-specific probes are available for identifying homologous chromosomes. This allowed to visualise chromosome-size differences, the extent of which varies according to the underlying mechanisms, their frequency and possibly their functional consequences. As such, three distinct molecular mechanisms have been described so far as being responsible for chromosome size-variation in trypanosomatids: (1) expansion/contraction of telomeric repeats are responsible for small size-variations (up to 35 kb) and they are reported to happen at a very high frequency, reaching an amplitude of 10 bp/division in *T. brucei* (Bernards et al. 1983). Functional significance of these phenomena is unknown, but in African trypanosomes, telomere exchange, does play a role in VSG gene switching (Rudenko et al. 1996); (2) amplification-deletions among tandemly repeated genes cause larger size-differences (up to 400 kb) as illustrated in *Leishmania* (Victoir et al. 1995, Inga et al. 1998, Kebede et al. 1999) and *T. cruzi* (Wagner & So 1990, Campetella et al. 1992, Aslund et al. 1994, Henriksson et al. 1995). As for telomeric sequences, size-variation is progressive, but its frequency is lower. In *Leishmania,* we found rearrangement among the gp63 gene locus (leading to chromosome size-polymorphism) in a strain cultivated over four years (Victoir et al. 1995), while in *Sacharomyces cerevisiae* the frequency of amplification of rDNA was evaluated at about $5x10^{-3}$ copies/generation (Szostak & Wu 1980). Considering the importance of the rearranged genes, functional consequences might be expected through (i) gene dosage (Ashburner 1989), (ii) deletion of unique interspersed genes (Bourke et al. 1996), and (iii) effect on intergenic regulatory sequences (Ramamoorthy et al. 1995); (3) fusion/fission events are responsible for the most significant size-differences. For instance in *Leishmania*, there is a difference of about 1,200 kb between the 8/29 fused chromosome of NWL and the individual chromosome 8 of OWL and NWV (as calculated from data of Britto et al. 1998). In contrast to previous mechanisms, fusion/fission is not characterised by stepwise size-variation. Functional consequences are unknown, but considering their low frequency (three in the whole genus *Leishmania*; Britto et al. 1998), they most probably reflect major evolutionary events.

A NEW METHOD FOR THE ANALYSIS OF CHROMOSOME-SIZE VARIATION: aCSDI

Interpretation of the extensive chromosome size-polymorphism among natural populations of parasites remains a main problem. Classically, processing of molecular data and building of trees are based on disjunctive encoding of all the character states (here the size of the different homologues), followed

by phenetic or cladistic analyses (Gower 1984). By doing so, any chromosome size-difference has the same weight in the analysis. However, as a consequence of the different mechanisms described above, the evolutionary importance of genomic rearrangements seems to vary proportionally to the extent of chromosome-size differences. The only theoretical exception is size-conservative rearrangements such as inversions, which – to our knowledge – are not described so far in trypanosomatids. We may thus assume that phenetic analysis of chromosome size-polymorphism should be based best on the weighing of size-difference, rather than on the disjunctive encoding. Therefore, we developed the measure of the absolute chromosome size difference index (aCSDI; Dujardin et al. 1995), in which the genomic distance between two organisms is simply the sum of the absolute size-differences between their homologous chromosomes. The formulation fits with the diploid state assumed for most chromosomes of trypanosomatids (Henriksson et al. 1990, Bogliolo et al. 1996, Britto et al. 1998), and is very close to the 'absolute genetic distance' of Gregorius (1984), which is considered as having one of the best mathematical properties (Katz 1988). After calculation of aCSDI, agglomeration may be performed by any algorithm like UPGMA (Sneath & Sokal 1973) or Fitch-Margoliash (Felsenstein 1984). This leads to significantly structured dendrograms in contrast to the ones built-up from disjunctively encoded data (Dujardin et al. 1998). We present hereafter the application of the aCSDI method to three evolutionary models.

INFRA-SPECIFIC EVOLUTION

The first application of aCSDI concerns analysis at low level of evolutionary divergence, in particular at inter- and intra-specific levels. *L. (V.) braziliensis*, one of the most aggressive species of Latin America, is endemic in the whole Amazonian basin (Guerra 1988). *L. (V.) peruviana* is a rather benign species, endemic only on the Pacific slopes of the Peruvian Andes and in some inter-Andean valleys, mostly in xerophytic environments (Guerra 1988). Despite these extensive phenotypic differences, the two parasites were shown to differ by only one out of 16 enzymatic loci (Bañuls et al. 2000), an indication of recent divergence (less than 1.5 Myrs, the estimated divergence time between *L. (L.) infantum* and *L. (L.) donovani*, two species differing by two enzymatic loci; Moreno et al. 1986). This even led some authors to question the validity of the distinction of the two species (Chouicha et al. 1997).

In order to better understand the evolutionary relationships between both parasites, an allopatric sampling was performed in their territory of endemism. Our sampling key was the bio-geographical units (BGUs) described in Peru on the basis of the endemism of butterfly species (Lamas 1982). Our working hypothesis was that eco-geographical factors responsible for structuring of butterfly species were also responsible for structuring among sand fly vectors and their parasites. Our assumption was supported by the known relationships existing between sand fly and environment (Cameron & Davies 1993). Five chromosomes (out of 35 in subgenus *Viannia*, Britto et al. 1998) were selected for their significant size-polymorphism and, analysed using the aCSDI method (Dujardin et al. 1995). All *L. (V.) braziliensis* isolates grouped together in a small cluster, at distance from *L. (V.) peruviana* (Fig. 1). The latter displayed a much higher chromosome size-polymorphism and its populations were structured according to their BGU of origin along a north-south cline. Northern *L. (V.) peruviana* isolates presented a higher karyotype similarity with *L. (V.) braziliensis* than with southern *L. (V.) peruviana*. Interestingly, the BGU where the northern *L. (V.) peruviana* isolates were collected is close to the pass of Porculla, the only natural pass in the Peruvian Andes between the Amazonian forest and the Pacific slopes. Considering a divergence time of *L. (V.) peruviana* and *L. (V.) braziliensis* inferior to 1.5 Myrs (see above), both species likely diverged after uplifting of the Andes (achieved 3 Myrs ago, Van der Hammen 1982). Accordingly, we think that this dynamic picture of karyotype variation reflects the evolution of *L. (V.) peruviana* from *L. (V.) braziliensis*, in the course of its colonisation of the Pacific slopes of the Andes, through the Porculla pass. Through North-South migration and isolation, *L. (V.) peruviana* would have increased its genetic and genomic differentiation during its journey through the various Pacific BGUs and their respective sand fly species (Davies et al. 1993, Villaseca et al. 1993, Caceres et al. submitted). This trend is corroborated by an important eco-epidemiological argument: evolution from sylvatic (reservoir: rodents and edentates for *L. (V.) braziliensis*; Guerra 1988) to domestic transmission (reservoir: dog for *L. (V.) peruviana*; Llanos-Cuentas et al. 1999). Furthermore, this trend was supported recently by genomic evidences: a set of *L. (V.) braziliensis*-specific genes (gp63, Victoir et al. 1998) was found to lack in *L. (V.) peruviana*. Considering the phylogenetic proximity of both species (Chouicha et al. 1997), deletion in *L. (V.) peruviana* is easier to explain than acquisition by sequence divergence in *L. (V.) braziliensis.*

Further studies established that the above-described chromosome size-polymorphism was indeed associated with significant rearrangements (at

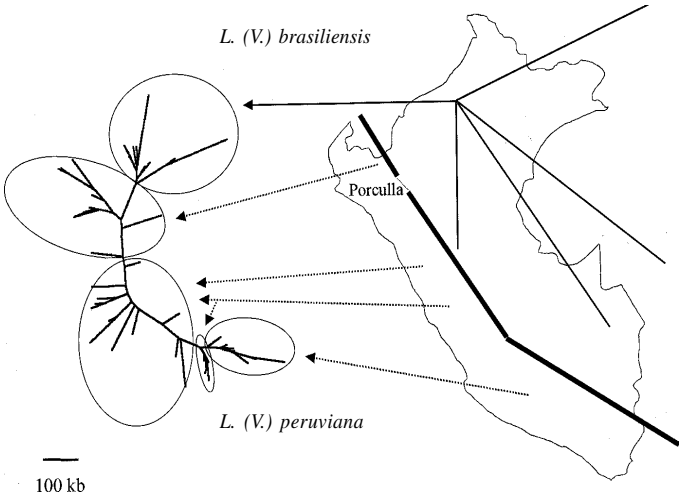

Fig. 1: size-polymorphism of chromosomes 2, 10, 11, 27 and 134Sg in Peruvian *Leishmania (V.) braziliensis* and *L. (V.) peruviana* isolates. Agglomeration by the Fitch-Margoliash method after aCSDI calculation. Porculla: natural pass between forest and Pacific slopes of the Andes (thick line)

least in four out five chromosomes studied) among important tandemly repeated genes. First, halving of gp63 gene copy number and deletion of a specific isogene were found to discriminate *L. (V.) peruviana* from *L. (V.) braziliensis* (Victoir et al. 1995). Secondly, decrease in copy number of rDNA (Inga et al. 1998), mini-exon (Kebede et al. 1999) and cystein proteinase b (Polet 1999) was shown to be involved in the North-South chromosome-size decrease observed within *L. (V.) peruviana*.

Our results thus clearly advocate at inter- and infra-specific levels that chromosome size-polymorphism (i) is reflecting gene rearrangements with a potential adaptive significance, (ii) allows a sensitive monitoring of genetic variation (if weighed by aCSDI), and (iii) may be used for inferring novel evolutionary hypotheses.

EVOLUTION WITHIN THE GENUS *LEISHMANIA*

In the *L. (V.) braziliensis-L. (V.) peruviana* model, species divergence is thought to be recent and consequences of gene rearrangements are still visible. Next question was to evaluate if this might still be valid at higher evolutionary level, or if the signal would become buried in the evolutionary noise. Therefore, we processed the data from the karyotypes of major *Leishmania* species published by Wincker et al. (1996) and Britto et al. (1998), and we calculated the size of each chromosome. It had been shown that, out of 36 chromosomes, 31 linkage groups were preserved across the genus, while five were the object of fusion/fission events (Britto et al. 1998).

We calculated aCSDI between each species, for the 31 "conserved" chromosomes. The ensuing dendrogram (Fig. 2) clearly showed a structuring into three clusters, corresponding to the three major taxonomical categories described by MLEE analysis within genus *Leishmania*: OWL, NWL and NWV. Interestingly, the genomic distance separating NWL from the two other clusters was quite high (about 1,400 kb). After analysis of individual chromosomes, it appeared that this distance corresponds essentially to a significantly lower size for 11 NWL chromosomes. Such a result should be confirmed by the analysis of additional stocks but, like in the *L. (V.) braziliensis-L. (V.) peruviana* model described above, it obviously raises the question about the nature of the sequences implicated in these size-differences. Considering the higher divergence time between NWL and OWL/NWV than between *L. (V.) braziliensis* and *L. (V.) peruviana*, a larger number of significant gene rearrangements might be expected. Accordingly, a specific attention should be paid on the gene content of these 11 chromosomes, as it might give further clues to the functional differences existing between the three major groups of *Leishmania*. This illustrates the feedback contribution of chromosome evolutionary studies for comparative genomics.

Thus, there is a good qualitative agreement between major evolutionary groups as defined by chromosome size-polymorphism and sequence polymorphism (as inferred by MLEE analysis). Quantitatively, however, relative distances between the three groups are not similar. Indeed, chromosome size analysis positioned NWL as the most

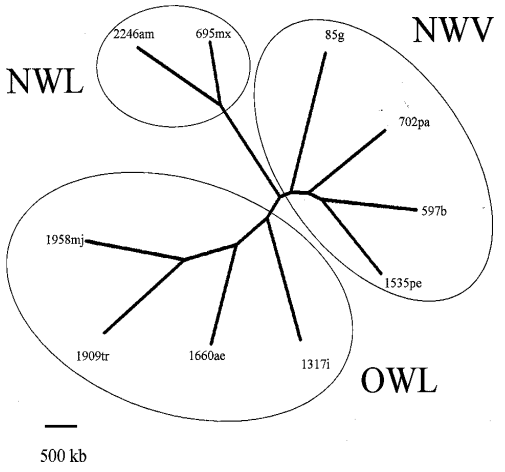

Fig. 2: size-polymorphism of 31 conserved chromosomes in genus *Leishmania* (from data of Britto et al. 1998 and Wincker et al. 1996) Agglomeration by the Fitch-Margoliash method after aCSDI calculation. NWV, subgenus *Viannia* (g, *L. (V.) guyanensis* ; pa, *L. (V.) panamensis*; b, *L. (V.) braziliensis*; pe, *L. (V.) peruviana*); NWL, New World subgenus *Leishmania* (am, *L. (L.) amzonensis* ; mx, *L. (L.) mexicana*); OWL, Old World *Leishmania* (mj, *L. (L.) major*; tr, *L. (L.)tropica*; ae, *L. (L.) aethiopica*; i, *L. (L.) infantum*); numbers correspond to LEM codes of Montpellier.

remote group within genus *Leishmania*, while MLEE analysis situated NWL and OWL close to each other (Thomaz-Soccol et al. 1993). This remote position of NWL was reinforced by counting the minimal number of fusion/fission events among the five chromosomes not considered by aCSDI analysis. While one event only was separating NWV and OWL, two and three events did separate NWL from OWL and NWV respectively (Britto et al. 1998). Two factors, inconstant mutation rates and/or selection might explain this relative incongruence. The Fitch-Margoliash dendrograms here presented do not consider a molecular clock hypothesis, but even when considering it (Kitch option in Phylip package), the remote position of NWL remained. Unfortunately, the test for molecular clock hypothesis as described by Felsenstein (1984) could not be performed because distances are not independent (on a same gel, chromosome sizes are evaluated from the same molecular marker). Selection was previously shown to play an important role in the modulation of chromosome size-variation in the *L. (V.) braziliensis-L. (V.) peruviana* model (Dujardin et al. 1998), and this hypothesis should be further explored at genus level by analysis of chromosome size-variants genetic content.

Our results thus show that, despite an extensive karyotype plasticity, chromosome size analysis does allow long range evolutionary studies at genus level. Potential outgroups such as *Sauroleishmania, Endotrypanum* or *Crithidia* should now be added, in order to better understand the evolutionary relationships within genus *Leishmania*; among others, this might allow to further document the Paleotropical (Kerr 2000) or Neotropical (Noyes 1998) origin of this genus. Further work is also required to understand the significance of the large genomic differences existing between major groups and to test their eventual adaptive value.

EVOLUTION WITHIN *T. CRUZI*

In the next step, our approach was applied to another organism, *T. cruzi.* This species has been extensively studied by MLEE, RAPD, and natural populations were found to be heterogeneous and highly structured (Brisse et al. 2000). Two major lineages were described, both being very heterogeneous. First lineage (clade 1) was relatively less heterogeneous, and the second one was further subdivided into five smaller lineages (designated clades 2a to 2e). Our aim was thus to evaluate whether chromosome size-polymorphism would also reflect this structuring. Therefore, seven chromosomes recognised by specific probes were analysed in representative stocks of the six *T. cruzi* major clades, and data were processed by aCSDI (Henriksson et al. in preparation). On the Fitch-Margoliash dendrogram (Fig. 3), all isolates (without exception) clustered according to their clade of origin. Furthermore, like with MLEE/RAPD, clade 1 was more homogeneous and, consistently with RAPD, quite distant from the other ones. Chromosome discrimination of the six clades lineages might have important biological or medical significance.

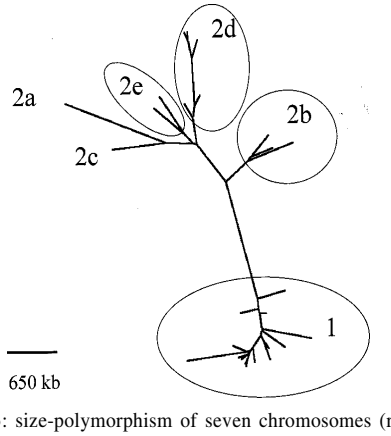

Fig. 3: size-polymorphism of seven chromosomes (recognised by probes 1F8, cruzipain, FFAg6, Tc2, CA7.12, CA7.32 and P19; Henriksson et al. 1995) in *Trypanosoma cruzi*. Agglomeration by the Fitch-Margoliash method after aCSDI calculation. Numbering of clades after Brisse et al. (2000)

Indeed, significant differences in clinically relevant biological parameters have been reported between isolates belonging to some of these clades (Montamat et al. 1996, Laurent et al. 1997, Revollo et al. 1998, De Lana et al. 1998). Identification of the genes involved in respective chromosome rearrangements will help exploring the molecular bases of these phenotypic differences.

Analysis of individual chromosomes showed a particular size-distribution. Indeed, in most chromosomes, a bi-modal distribution was observed, with important size-differences between the respective modes (Fig. 4). The stepwise size-variation observed within each mode is suggestive of amplification/deletion among tandemly repeated genes, as shown for *Leishmania*. In contrast, the transition from one mode to the other was not stepwise, and should correspond to discrete but less frequent evolutionary events, like translocation or deletion of large chromosome arms. The occurrence of such mechanisms was supported by the linkage of two markers (CA7.12 and 7.32) on a same chromosome in clade 1, but not in the other clades (Henriksson et al. 1995).

Chromosome analysis of *T. cruzi* allowed to highlight the relativity of taxonomical subdivisions in trypanosomatids. Indeed, in *T. cruzi*, the maximal aCSDI value measured (for seven chromosomes) between the two major lineages was 3,500 kb (average of 500 kb/chromosome). In the whole genus *Leishmania*, the maximal aCSDI value (for 31 chromosomes) was of 4,750 kb (average of 150 kb/chromosome). This result can be accounted for by either a higher mutation rate or a higher frequency of large rearrangements in the species *T. cruzi*, than in genus *Leishmania*. However, when other genetic characters were considered, like RAPD and MLEE, the same picture was encountered (Bañuls et al. 1999). It appears thus more likely that species definition should be questioned in both cases. In the absence of strict applicability of the sexual exchange criterion (Mayr 1969) to trypanosomatids because of their essentially clonal structure (Tibayrenc et al. 1990), species definition is basically operational only. Our results clearly show that chromosome analysis contributes significantly to the definition of the major evolutionary groups among trypanosomatids, and thus could contribute with other genetic characters for re-definition of corresponding taxonomic units.

CONCLUSIONS AND PROSPECTS

Trypanosomatids are characterised by extensive genomic plasticity. With the appropriate algorithm (aCSDI), it is possible to rely on chromosome size-polymorphism to infer hypotheses at different evolutionary levels. Qualitative congruence with hypotheses built-up from DNA sequence polymorphism (as inferred here from MLEE and RAPD data) contributed to validate our approach. At low evolutionary level chromosome size-polymorphism involves rearrangement of key genes, with important adaptive value. The same is occurring likely at higher evolutionary levels, where dramatic chromosome size-variations are observed. This should stimulate interaction between evolutionary studies like here presented and parasite genome sequencing projects. On one hand, identification of major evolutionary groups should call for peculiar attention to the genic content of chromosomes responsible for major structuring, and to their potential relationship with biological (including medical) differences. This might be one of the major application of evolutionary studies. On the other hand, definition of linkage groups should be used for performing direct gene rearrangement studies. This is important for further validating indirect studies based on chromosome size-polymorphism, but also for inferring hypotheses at higher evolutionary levels, where chromosome size might not be applicable anymore. It is too early to propose – like in prokaryotes – that the map of trypanosomatid cells is in their chromosomal organisation. However, our results clearly demonstrate that chromosomes together with gene sequences offer useful maps for finding our way in trypanosomatids' evolution.

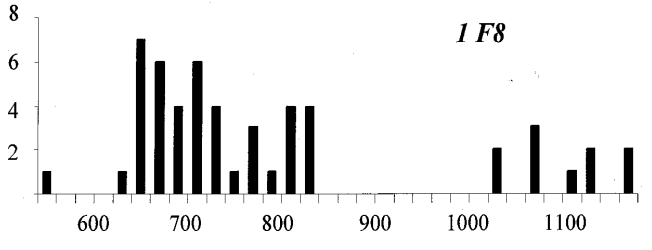

Fig. 4: bi-modal size distribution of 1F8 chromosome in a sample of *Trypanosoma cruzi* stocks representing the six major clades. Size is expressed in kb.

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To Dr Jamie Stevens who organised The Trypanosome Evolution Workshop and stimulated the writing of present review.

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CHAPTER 3

Isolation and molecular identification of *Leishmania (Viannia) peruviana* from naturally infected *Lutzomyia peruensis* (Diptera: Psychodidae) in the Peruvian Andes

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Isolation and molecular identification of *Leishmania* (*Viannia*) *peruviana* from naturally infected *Lutzomyia peruensis* (Diptera: Psychodidae) in the Peruvian Andes

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Leishmania (Viannia) peruviana *was isolated from 1/75* Lutzomyia peruensis *captured during May 2006 in an endemic cutaneous leishmaniasis region of the Peruvian Andes (Chaute, Huarochiri, Lima, Peru). Sand fly gut with promastigotes was inoculated into a hamster and the remaining body was fixed in ethanol.* L. (Viannia) *sp. was determined by polymerase chain reaction (PCR), and* Leishmania *species through molecular genotyping by PCR-restriction fragment length polymorphism analyses targeting the genes* cpb *and* hsp70*, resulting* L. (V.) peruviana*. The infected sand fly appeared 15 days after the rains finished, time expected and useful real time data for interventions when transmission is occurring.*

Key words: *Leishmania peruviana - Lutzomyia peruensis* - polymerase chain reaction-restriction fragment length polymorphism - Peru

An important step for the incrimmination of *Leishmania* vectors is the report of naturally infected sand fly species (Killick-Kendrick & Ward 1981). The proportion of sand flies infected with *Leishmania* in places frequented by humans is taken into account. Several methods have been applied for this requirement, being the sand fly gut dissection first used, although it is the less productive one because of the low *Leishmania* infection rate in sand flies (0.001-2.6) (Perez et al. 1994), the requirement of living sand flies and well trained personnel for sand fly gut dissection. To date this technique is still being used with the purpose of isolating the parasite from an individual sand fly. Sand flies can also be cryopreserved in liquid nitrogen to be dissected months later. Young et al. (1987) dissected 18,463 sand flies, obtaining 11 (0.059%) with *Leishmania* promastigotes in Colombia. In another technique, the whole sand fly body is homogenized in pools of up to 24 individuals and then inoculated into hamsters, where *Leishmania* infection produces lesions in at least six weeks. Finally, the parasites are isolated by aspiration of the lesions and transferred to culture medium. Using this technique the number of infected sand fly individuals is unknown and it is possible to have a mixture of *Leishmania* strains in a single pool. Sentinel hamsters have also been used to obtain *Leishmania* strains from sand flies (Herrer 1982a). The animals are exposed to sand fly bites in endemic areas and then aspirates of different parts of the hamster body are taken and inoculated into culture medium to isolate the parasites. Here indeed, the infected sand fly species are unknown. Detection of *Leishmania* DNA is now a commonly used technique. Sand flies can be preserved dried or frozen and large number of sand flies can be processed individually or in pools. By means of polymerase chain reaction (PCR) it is possible to amplify and detect DNA of less than one *Leishmania* parasite (Lopez et al. 1993). *Leishmania* species identification is now done by using specific primers designed for different *Leishmania* genes currently used as targets for molecular genotyping (Garcia et al. 2004, 2005).

The search for *Leishmania* vectors in Peru started in the Andes, where the diversity of sand fly species is low (1-6 species in a single valley). Cruzado (1987) reported four specimens of *Lutzomyia peruensis* infected with *Leishmania* in La Libertad. Herrer (1982b) obtained two (2.06%) isolates of *Leishmania* sp. from 97 specimens of *Lu. peruensis* homogenized and inoculated into hamsters, in the Rimac Valley (Lima). Perez et al. (1991) reported two isolations of *Leishmania* from sand flies, the first (strain IPRN/PE/87/Lp52) by inoculation of pools of homogenized *Lu. peruensis* (0.34%) and the second in a sentinel hamster, in the Huayllacallan Valley (Ancash). The strain Lp52 has been recently identified as *L. (Viannia) guyanensis* by means of PCR-restriction fragment length polymorphism (RFLP) analyses of kDNA minicircles (kDNAPCR-RFLP) (Callapiña 2001). Perez et al. (1994) worked with pools of 4-10 sand fly individuals for PCR detection of DNA of the *L. (Viannia)* subgenus, and found two pools of *Lu. peruensis* (0.2%) and six of *Lu. verrucarum* (0.13%) positive for *L. (Viannia)* sp. Caceres et al. (2002) in Ayacucho dissected 1849 *Lu. ayacuchensis*, obtaining five (0.27%) positives for *Leishmania* sp. by PCR, and four of which were cultured and determined as *L. peruviana* by multilocus enzyme electrophoresis and molecular karyotyping.

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The main goal of the present study was to find infected sand flies when they are most numerous, which is after the rainy period in the Andes, from the end of April to May (Perez et al. 1994), and also to carry out the *Leishmania* species determination through PCR amplification, followed by PCR-RFLP.

Sand flies for this study were captured in Chaute (Huarochirí, Lima), SL11º56.455' and WL76º30.392', at 2400 m of altitud, in May 2006, using a Shannon trap with protected human bait from 18:00 to 19:00 (Perez et al. 1988). All sand flies captured were placed in collecting flasks and provided with sugar solution (30%) in a piece of cotton, and transported to our laboratories in Lima where living sand flies are maintained in a sand fly colony room at constant temperature of 20ºC.

San dfly guts were dissected in saline solution with antibiotics and observed under compound microscope at 400x magnification. A total of 317 female sand flies were dissected in May 2006 (75 of *Lu. peruensis* and 242 of *Lu. verrucarum*).

A typical peripylarian infection was detected in a 1/75 *Lu. peruensis* captured by JE Perez on May 6, 2006. The stomodeal valve and midgut were full of promastigotes and traces of digested blood, the peritrophic membrane was not observed, the pylorus showed pear shaped promastigotes attached to its interior walls and no free promastigotes were observed in the hind gut. The infection was apparently old (6 to 9 days), the infected sand fly was attracted to the human bait, probably to take a bloodmeal. The gut was placed in another drop of saline solution with antibiotics, broken to release the parasites and then inoculated onto 5% blood agar base biphasic culture medium, and also inoculated onto the hind feet of a golden hamster (*Mesocricetus auratus*). The rest of the sand fly body (head, thorax, and abdomen) was fixed in absolute ethanol and processed for *Leishmania* DNA extraction, PCR using the primers MP1-L and MP3-H, which are specific for *L. (Viannia)* spp. (Arevalo et al. 1993), and molecular genotyping by PCR-RFLP using two different genes as targets, which encode major *Leishmania* antigens cysteine proteinase B (*cpb*), and heat shock protein 70 (*hsp70*) (Garcia et al. 2004, 2005).

Although the cultures were heavily contaminated, they were processed for amplification of *Leishmania* DNA. After seven weeks, the hamster did not show lesion, just a weak swelling and reddish area at the inoculation sites. The abnormal sites were aspirated and this material inoculated into culture medium, and maintained at 23ºC. *Leishmania* promastigotes were observed on the 3rd day of culture and the strain was coded IPRN/PE/2006/Chaute1.

Leishmania DNA amplification was successful from all materials which include fragments of sand fly body, contaminated culture and the positive culture from hamster lesions. The PCR employed, using the primers MP1-L and MP3-H, showed that the DNA isolated from the strain IPRN/PE/2006/Chaute1 belonged to any of the species belonging to the subgenus *L. (Viannia)*, where the characteristic amplification products of 72 base pair was shown by all materials (Arevalo et al. 1993). This fact was confirmed through a PCR technique using *cpb* gene (Fig. 1), the species of the subgenus *L. (Viannia)* including the strain IPRN/PE/2006/Chaute1 shown a similar pattern, a band of 1170 bp. The PCR-RFLP technique applied afterwards showed, using two different markers (*cpb* and *hsp70*), that the parasite was in fact *L.* (*V.*) *peruviana* (Figs 2, 3). Using the *cpb* gene alone, which shows a characteristic pattern for *L. (V.) braziliensis* after the restriction enzyme analysis with Taq1, we obtained a similar pattern to the reference strains *L. (V.) peruviana* (MHOM/PE/90/HB22) and *L. (V.) guyanensis* (IPRN/PE/87/Lp52) (Garcia et al. 2005). Using the *hsp70* gene as marker, the result is a specific pattern for *L. (V.) guyanensis*, the pattern obtained after the restriction enzyme analysis with BsuRI showed similarity with the reference strains *L. (V.) peruviana* (MHOM/PE/90/HB22) and *L. (V.) braziliensis* (MHOM/PE/93/LC2177); the identity of these strains was confirmed by Garcia et al. (2004, 2005).

The combination of the presence of 343 bp for the *cpb* gene band of *L. (V.) braziliensis*, and the absence of a 224 bp for the *hsp70* gene band of *L. (V.) guyanensis*, distinguishes *L. (V.) peruviana* from these two species (cf. Garcia et al. 2005).

The isolation of the strain Chaute1 was carried out within the peak season of sand fly abundance, *Leishmania* infection both of sand flies and humans in Chaute. This situation was previously described by Perez et al. (1994), and it occurs seasonally just after the rains finish in the Andes (late April). The obtention of infected

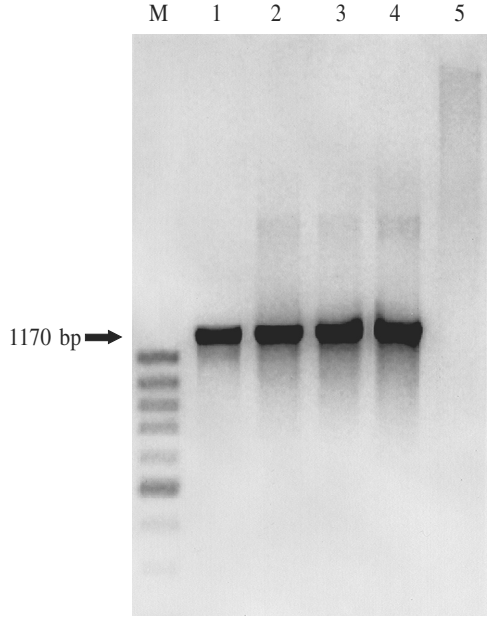

Fig. 1: agarose electrophoresis gel of *cpb* polymerase chain reaction products. M: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (IPRN/PE/87/Lp52); 5: negative control.

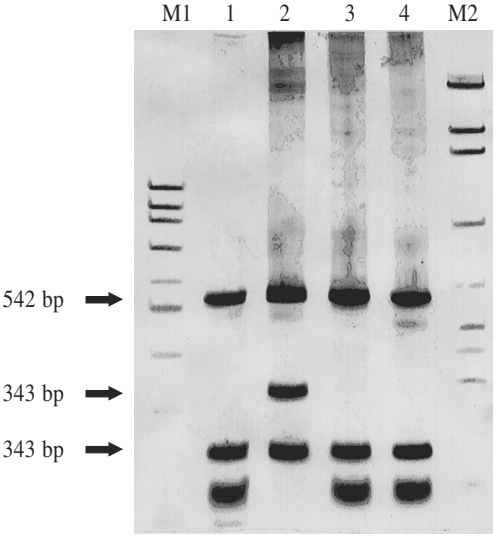

Fig. 2: *cpb* polymerase chain reaction-restriction fragment length polymorphism analyses patterns (Taq1) after polyacrylamide gel electrophoresis. M1: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (IPRN/PE/87/Lp52); M2: pGEM ladder.

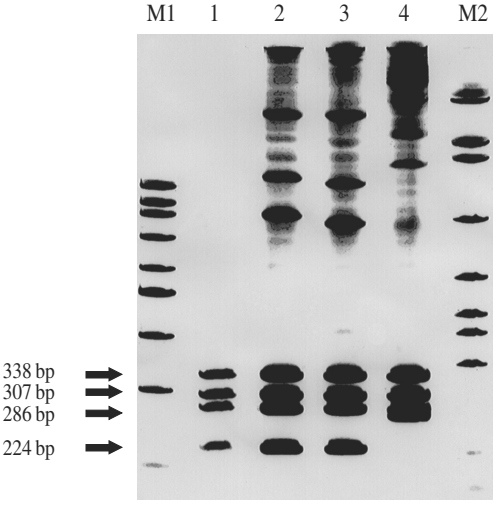

Fig. 3: *hsp70* polymerase chain reaction-restriction fragment length polymorphism analyses patterns (BsuRI) after polyacrylamide gel electrophoresis. M1: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (Lp52); M2: pGEM ladder.

sand flies is more successful on this time of the year in the Andean *Leishmania* endemic areas. Sand flies are also a nuisance insect, an average of 472 sand fly bitings can be received by night in Chaute in the sand fly peak season.

It is understood that the chance to find naturally infected sand flies with *Leishmania* is normally very low, but is necessary to obtain living pathogens from sand flies for further studies. It is convenient to determine if the *Leishmania* strains proceeding from sand flies and animals are the same as those proceeding from humans. Studies like that should be performed with some periodicity as surveillance to detect changes in the genetical patterns (infectivity, virulence) of *Leishmania* species strains through time in a given endemic area. *Leishmania* infection rate of animals, which are the source of infection of sand flies, is variable, it is 0.2-2.8% in wild mice and 2.6-11.9% in dogs (Llanos-Cuentas et al. 1999). The isolation of the strain Chaute1 represents 1.33% of the *Lu. peruensis* captured in May 2006 in Chaute, the rate found is higher than the records in other Andean areas (Caceres et al. 2002).

L. verrucarum was not found infected with *Leishmania* in this study, which contrast with the findings of Perez et al. (1994), where six infected individuals were detected by means of PCR. This fact probably correspond to light infections probably missing in the observation under microscope and the fact that PCR technique is much more effective for the detection of early infections and those that may not succeed in the sand fly. This is the main reason why it is highly recommended.

The study of *Lu. peruensis* becomes relevant. This species coexist with *Lu. verrucarum* and both species are vectors of Andean cutaneous leishmaniasis which is caused mainly by *L. (V.) peruviana.* In the Department of Ancash (central Peruvian Andes), *L. (V.) guyanensis* also affects the human population in the same areas, being the second *Leishmania* species found in *Lu. peruensis* (Perez et al. 1994).

Information about infected sand flies can be available in few days combining sand fly gut dissection with PCR detection of *Leishmania* DNA and *Leishmania* species identification by PCR-RFLP. Sand fly dissectors should work routinarely closer to PCR procedures equipped with a set of specific primers. The knowledge of the *Leishmania* and its vector species in a real time can result in appropriate intervention when the transmission is taking place in a given area.

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CHAPTER 4

Evaluation of an in vitro and in vivo model for experimental infection with *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana*

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Evaluation of an *in vitro* and *in vivo* model for experimental infection with *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana*

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SUMMARY

Leishmania (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana* are two parasite species characterized by a very different pathogenicity in humans despite a high genetic similarity. We hypothesized previously that *L.* (*V.*) *peruviana* would descend from *L.* (*V.*) *braziliensis* and would have acquired its '*peruviana*' character during the southward colonization and adaptation of the transmission cycle in the Peruvian Andes. In order to have a first appreciation of the differences in virulence between both species, we evaluated an *in vitro* and *in vivo* model for experimental infection. A procedure was adapted to enrich culture forms in infective stages and the purified metacyclics were used to infect macrophage cell lines and golden hamsters. The models were tested with 2 representative strains of *L.* (*V.*) *braziliensis* from cutaneous and mucosal origin respectively and 2 representative strains of *L.* (*V.*) *peruviana* from Northern and Southern Peru respectively. Our models were reproducible and sensitive enough to detect phenotypic differences among strains. We showed *in vitro* as well as *in vivo* that the *L.* (*V.*) *braziliensis* was more infective than *L.* (*V.*) *peruviana*. Furthermore, we found that *in vitro* infectivity patterns of the 4 strains analysed, were in agreement with the geographical structuring of parasite populations demonstrated in our previous studies. Further work is needed to confirm our results with more strains of different geographical origin and their specific clinical outcome. However, our data open new perspectives for understanding the process of speciation in *Leishmania* and its implications in terms of pathogenicity.

Key words: *Leishmania* (*V.*) *braziliensis*, *Leishmania* (*V.*) *peruviana*, infectivity, virulence, macrophage, hamster.

INTRODUCTION

The genus *Leishmania* contains parasite species causing a spectrum of clinical phenotypes in humans. Understanding the factors that underlie this pleomorphism is important for the design of rational diagnosis and surveillance strategies, as well as for the development of new drugs and vaccines. Obviously, parasite and host factors interact in producing a clinical phenotype (de Almeida *et al.* 2003) and their elucidation requires, among others, good natural and experimental models.

The 2 neotropical species *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana* fit perfectly in this context. The former is the most aggressive species of subgenus *Viannia*, causing severe cutaneous and mucosal lesions, in patients throughout the Amazonian basin (Guerra, 1988). In contrast, *L.* (*V.*) *peruviana* causes benign cutaneous lesions and it was never found to be associated with the mucosal phenotype (Lucas *et al.* 1998). To date, these species have only been encountered in Peru, being (essentially) endemic in the Andean highlands (between 800 and 3000 metres above sea level). Despite these major clinical differences, the 2 parasites are genetically very similar: a few markers only allow their discrimination (Dujardin *et al.* 1995*a*; Victoir *et al.* 1995; Bañuls *et al.* 2000; Garcia *et al.* 2005; Zhang *et al.* 2006). On the basis of karyotype data, it has been hypothesized that *L.* (*V.*) *peruviana* would descend from *L.* (*V.*) *braziliensis* and would have acquired its 'peruviana' character during the southward colonization of the transmission cycle in the Peruvian Andes (Dujardin *et al.* 1993, 1995*a*, *b*, 1998, 2000, 2002).

Molecular studies showed that some of the genetic characters distinguishing both species concerned genes encoding putative virulence factors (virulence

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being defined as the degree of pathogenicity of a microorganism genetically endowed with that capacity, as manifested against a host with an intact immune system under normal conditions; Chang *et al*. 2003): the metalloprotease gp63 (Victoir *et al*. 1995) and the cysteine proteinase b (Garcia *et al*. 2005). This might support the contribution of parasite factors to the difference in pathogenicity observed in humans. However, these molecular markers might be confounding factors and further documentation of the role of the parasite and host should come from experimental studies *in vitro* and *in vivo* but these were mainly done for *Leishmania* strains from subgenus *Leishmania* (mostly *L*. (*L*.) *major*) (Garin *et al*. 2001; Achour *et al*. 2002; Baldwin *et al*. 2003; Vladimirov *et al*. 2003; Sádlová *et al*. 2006).

In the present study, we developed *in vitro* and *in vivo* models for experimental infections and compared the phenotypic differences between *L*. (*V*.) *braziliensis* and *L*. (*V*.) *peruviana* strains. To that end, we selected 4 clinical isolates from Peru: (i) 2 representative strains of *L*. (*V*.) *braziliensis* isolated from patients with cutaneous and mucosal lesions, respectively and (ii) 2 representative strains of *L*. (*V*.) *peruviana* originating from the North and the South of the Andean endemic area. A key issue for this type of experimental study is to work with populations that are as homogenous as possible. Therefore, a procedure was adapted to enrich infective-stage parasites (metacyclics) in culture. These purified parasites (homogeneous population) were used to infect macrophage cell lines as well as golden hamsters. We showed *in vitro* as well as *in vivo* that under our experimental conditions the *L*. (*V*.) *braziliensis* isolates used here were more infective than the *L*. (*V*.) *peruviana* ones.

MATERIALS AND METHODS

Leishmania *strains*

The 4 representative strains used in the present study were isolated from Peruvian patients with cutaneous or mucosal lesions. The *L*. (*V*.) *braziliensis* were sympatric isolates originating from the Amazonian basin: MHOM/PE/91/LC2043 (mucosal origin), and MHOM/PE/91/LC2177 (cutaneous origin); the *L*. (*V*.) *peruviana* isolates were both of cutaneous origin: MHOM/PE/90/HB86 (Andes from the North) and MHOM/PE/90/LCA08 (Andes from the South). In order to ensure the homogeneity of the parasite population, the strains were cloned by the micro-drop method (Van Meirvenne *et al*. 1975) and characterized by multi-locus enzyme electrophoresis (13 enzymes, Bañuls, 1998). Identity of the strains was confirmed by Pulsed Field Gradient Electrophoresis before the beginning of the experiments, as described elsewhere (Dujardin *et al*. 1987). For each strain, cryostabilates made from parasites with a minimum number of sub-inoculations were thawed and first cultivated at 26 °C in a biphasic agar medium supplemented with 15% defibrinated rabbit blood and 0·85% saline solution. They were then adapted to grow at 26 °C in an enriched medium, M199 (Sigma), supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS) and adjusted to pH 7·4. In order to have enough parasites for the experiments, promastigotes at early stationary phase (1×10^6 parasites/ml) were subcultivated into 2 bottles (50 ml each) with medium M199, one at pH7·4 and the other adjusted to pH 5·5 (modified method by Bates and Tetley, 1993; Zakai *et al*. 1998; Almeida *et al*. 1993), and both were incubated at 26 °C. The growth curve was monitored by daily counting of parasites using a Neubauer brightline haemocytometer.

Ficoll centrifugation gradient

In order to obtain purified metacyclic parasites from the pH 5·5 culture, we adapted the method described by Späth and Beverley (2001). Ficoll Type 400 (Sigma) was used to prepare a 20% stock solution. The gradient was formed using Ficoll 20% at the bottom and Ficoll 10% on the top of a 15 ml or 50 ml tube, according to the volume of parasite culture to be purified. The parasite culture at early stationary phase (4th day of culture, according to the growth curve) was resuspended in M199 (2–6 ml according to the parasite concentration) and poured slowly on the top of the Ficoll 10% and centrifugated at 2500 rpm for 15 min. After centrifugation, the fraction above or inside the Ficoll 10% was re-covered, and an aliquot was observed under the microscope to count and verify the morphology and viability of the parasites. The remaining fraction of the purified parasites was used to evaluate their resistance to complement, and their infectivity *in vitro* and *in vivo*. From now on, these purified parasites will be called 'Met-pH5·5'. The metacyclic population has become relatively more homogeneous in comparison with the stationary promastigotes where mixed populations coexist. The homogenization process achieved by the Ficoll gradient centrifugation experiment facilitates separation of the parasites based on the physical properties: body and flagellum size, body shape (characteristics of the metacyclic forms) (Bates and Tetley, 1993).

Complement lysis test

Resistance to complement lysis of the Met-pH5·5 parasites and promastigotes obtained from stationary phase culture at pH 7·4 (4th day according to the growth curve, and from now on called 'Pro-pH7·4') was tested. The parasites (3×10^6) were exposed to serial dilutions of human serum from a healthy

individual (from 1 : 1 to 1 : 128 in phosphate buffer solution). In order to estimate the percentage of surviving parasites, we counted in duplicate the parasites showing flagellar movement using a Neubauer brightline haemocytometer. With the program Probit SPSS 3.2, the concentration of serum that produces 50% parasite lysis (LD_{50}) was estimated. This experiment was carried out in duplicate to ensure the reproducibility of the counting.

In vitro *infections*

For the *in vitro* infections 3 independent experiments were performed using the macrophage cell line RAW 264 (murine origin), which were infected with the Met-pH5·5 or Pro-pH7·4 parasites in a ratio of 30 : 1 (parasites : macrophage). After 2 h incubation at 34 °C and 5% CO_2, free parasites were removed by repeated washings and the cultures were incubated for an additional 3 days at 34 °C and 5% CO_2. The infection was monitored (24 h, 48 h and 72 h post-infection) using parallel culture plates which were fixed with 0·25% glutaraldehyde and stained with Giemsa and May-Grünwald (dilution 1 : 4) in order to visualize the intracellular amastigotes. Up to 200 cells were counted in order to determinate the percentage of infected macrophages and the average number of amastigotes by infected macrophages. The product of the 2 previous parameters was used to define an infection index.

In vivo *infections*

In this case, for each species, we used the strain with the higher rate of *in vitro* infectivity. For each strain, 15 male golden hamsters (*Mesocricetus auratus*) of 4 weeks age, obtained from the Peruvian National Institute of Health, were randomly distributed in 3 groups, 5 animals in each, (i) one group inoculated at the right footpad with 1×10^6 promastigotes at stationary phase (Pro-pH7·4) resuspended in 100 μl of saline solution, (ii) the second group inoculated with 1×10^6 purified metacyclic promastigotes (Met-pH5·5) resuspended in 100 μl of saline solution and (iii) the third group inoculated at the left footpad with 100 μl of saline solution as a control. The diameter of the lesions was measured with a micrometer (0–25 mm), with a precision of 0·01 mm, every 2 weeks at the beginning of the experiment, and every week later on.

Statistical analysis

The data were reported as the average ± S.D. and using the PROBIT program to calculate the LD_{50} for the complement test. The *in vitro* and *in vivo* data did not have a normal distribution, so a non-parametric test i.e. the Two-sample Wilcoxon Rank-sum (Mann-Whitney) was used for comparing both *in vitro* and *in vivo* data. The differences were considered as statistically significant at $P < 0{\cdot}05$.

RESULTS

Enrichment and purification of metacyclics

For the 4 representative strains used here, enrichment and purification of metacyclics parasite forms (by parasite culture at pH 5·5 and using the Ficoll centrifugation gradient, respectively) were verified by comparing 3 parameters between Pro-pH7·4 and Met-pH5·5 parasites (Table 1). (1) The complement lysis test showed that twice as many Met-pH5·5 parasites survived serum concentrations compared to Pro-pH7·4 parasites. (2) Macrophage *in vitro* infection showed infection indices that were mostly higher for Met-pH5·5 parasites, and that the effect was more pronounced for the 48 h and 72 h infection groups, with differences that were statistically significant for all strains except for LCA08 at 48 h post-infection (95% confidence interval). (3) *In vivo* infection of hamsters revealed that lesions induced by Met-pH5·5 parasites were larger than those induced by Pro-pH7·4 parasites. For LC2043, differences were statistically significant (ANOVA, $P < 0{\cdot}05$) from the 2nd week post-infection until the end of the experiment (6 weeks follow up). Metacyclics of HB86 also showed significant differences at the 14th week post-infection (ANOVA, $P < 0{\cdot}05$). The maximum difference between Met-pH5·5 and Pro-pH7·4 size lesion was 3·16 mm (5th week post-infection) and 0·70 mm (14th week post-infection) for LC2043 and HB86 respectively. The difference between lesion pattern produced by Pro-pH7·4 and Met-pH5·5 parasites was larger in *L.* (*V.*) *braziliensis* than in *L.* (*V.*) *peruviana*, which could be explained by a higher proportion of metacyclics in Pro-pH7·4 parasites of HB86 used for the infection than the corresponding parasites of LC2043.

Infection differences between L. (V.) braziliensis *and* L. (V.) peruviana *representative strains*

To compare virulence between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*, we compared *in vitro* and *in vivo* results obtained with Met-pH5·5 parasites from the representative strains assayed here. These representative strains were chosen because of their genomic and genetic characteristics very well described elsewhere (Dujardin *et al.* 1995*b*; Bañuls *et al.* 2000). (i) The *in vitro* infectivity was expressed as the infection index rate at 24 h, 48 h, and 72 h post-infection of each representative strain. In all 3 independent experiments for each strain, the 4 strains ranked as following (from high to low): LC2043, LC2177, HB86 and LCA08 (Table 1 and

Table 1. Results for *in vitro* (panel A) and *in vivo* (panel B) infections using stationary non-purified promastigotes (Pro-pH7·4) and purified metacyclics (Met-pH5·5) from *Leishmania* (*Viannia*) *braziliensis* (Lb) and *L.* (*Viannia*) *peruviana* (Lp)

(*Program Probit SPSS 3.2 used to calculate the LD_{50} for the complement test. ** Differences in the *in vitro* infections using 95% confidence interval. *** Differences statistically significant in the *in vivo* infections (ANOVA; $P<0{\cdot}05$, $n=5$).)

Panel A.

		Enrichment metacyclics			*In vitro* infections (Infection index)					
		Serum % producing 50% lysis (95% confidence*)			24 h		48 h		72 h	
Strain		Pro-pH7·4	Met-pH5·5	Fold-time increased	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5
Lb	LC2043	0·059	0·16	2·7	170·43±6·74**	232·06±8·8**	235·78±8·36**	457·99±14·08**	368·37±12·77**	563·04±15·90**
Lb	LC2177	0·069	0·153	2·2	165·00±6·37	160·59±5·83	231·12±8·19**	301·96±9·3**	366·03±13·26**	491·6±15·04**
Lp	HB86	0·056	0·107	1·91	106·64±4·15**	148·11±5·9**	190·92±6·53**	236·74±8·22**	281·41±9·55**	379·5±13·63**
Lp	LCA08	0·069	0·136	1·97	86·66±3·38	97·62±3·87	150·54±5·28	148·25±6·07	136·78±5·06**	188·46±6·5**

Panel B.

	Time post-infection (*in vivo*)									
Strain	2 weeks***		3 weeks***		4 weeks***		5 weeks***		6 weeks***	
Lb	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5
LC2043	0±0	2·21±0·56	0·13±0·12	3·29±0·48	0·25±0·21	3·24±0·54	0·16±0·19	3·3±0·31	0·21±0·36	2·77±0·24
	11 weeks		12 weeks		13 weeks		14 weeks***			
Lp	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5		
HB86	0·44±0·48	0·92±0·12	0·75±0·40	1·1±0·09	0·99±0·57	1·6±0·66	1·06±0·24	1·76±0·47		

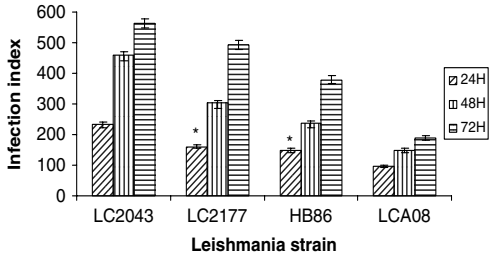

Fig. 1. *In vitro* infection of macrophages with purified metacyclic promastigotes: infection indices at different times post-infection for the *L.* (*V.*) *braziliensis* (LC2043 and LC2177) and *L.* (*V.*) *peruviana* (HB86 and LCA08) strains. For a given time-point, differences between strains were statistically different ($P=0{\cdot}000$, Mann-Whitney test) except for LC2177 and HB86 at 24 h (*).

Fig. 1). Noteworthy was that, despite the fact that the experiments were carried out with different culture batches of parasites and macrophages, the *in vitro* infection parameters were quite reproducible. Thus, for example, the average number of amastigotes per macrophage followed the same trend, at 72 h the values were $6{\cdot}83\pm0{\cdot}73$ (LC2043), $6{\cdot}3\pm0{\cdot}26$ (LC2177), $5{\cdot}3\pm0{\cdot}26$ (HB86) and $3{\cdot}2\pm0{\cdot}26$ (LCA08). A similar trend was observed with the percentage of infected macrophages (data not shown). Statistically, infection indices differed significantly between the 4 strains. This was observed at different times except for LC2177 and HB86 at 24 h post-infection (95% confidence interval). (ii) The *in vivo* infection experimental model was carried out with the more infective representative strain from each species observed after the *in vitro* experiments. In the case of the LC2043-infected animals, the development of the lesion was faster (2 weeks post-infection) than in the animals infected with HB86 (14 weeks post-infection). In addition, LC2043 lesions were larger in size than those produced by HB86 (maximum size lesion: 3·32 mm and 1·76 mm respectively) (Fig. 2A and B respectively). To assure that the lesion was due to the presence of the parasite, an aspirate from the lesion was taken after 1 month of infection and placed on blood agar medium, cultures were positives after 1 week of incubation (data not shown).

DISCUSSION

In the presented work, an experimental model was adapted and evaluated for analysing the *in vitro* and *in vivo* infectivity of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* representative strains. These two parasite species cause dramatically different pathologies in humans, despite their great genetic similarity.

The use of purified metacyclic promastigotes is very important for infectivity studies, not only because they indeed represent the infective forms inoculated by the vector, but also because they represent a well-defined and relatively homogeneous population. Such a population is essential to compare different *Leishmania* strains at a corresponding developmental stage. So far, most studies on metacyclogenesis have focused on parasites of subgenus *Leishmania*: *L.* (*L.*) *major*, *L.* (*L.*) *donovani*, *L.* (*L.*) *tropica*, *L.* (*L.*) *infantum* and *L.* (*L.*) *mexicana* (Bates and Tetley, 1993; Zakai *et al.* 1998). In comparison there are fewer reports on parasites of the subgenus *Viannia* (Almeida *et al.* 1993; Pinto-da-Silva *et al.* 2002), despite the fact that the latter comprise some species highly pathogenic for humans. Our experimental procedure was adapted from that evaluated in subgenus *Leishmania* species and combined the acidic induction of metacyclogenesis (Bates and Tetley, 1993; Zakai *et al.* 1998) with the purification of metacyclics by a Ficoll gradient centrifugation method (Späth and Beverley, 2001). Production of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* metacyclics was verified by measuring 3 parameters (and comparison with non-purified stationary promastigotes): (i) increased resistance to complement lysis, (ii) higher infection indices in macrophages and (iii) larger lesions in hamsters. All parameters indicated an homogeneous metacyclic population in the strains tested here. Previous work done by our group identified a molecular marker (the infective-insect stage protein also known as the Meta 1 protein) using metacyclic-enriched preparations (without purification) of *L.* (*V.*) *braziliensis* LC2043 (also produced by acid induction, Gamboa *et al.* 2007), one of the strains here studied. This marker, and maybe others identified in the future, might be studied by measuring their expression profile using purified preparations of metacyclic parasites from this and other *Leishmania* species from subgenus *Viannia* using the methodology described here (manuscript in preparation).

Using purified metacyclics as sources of infection, comparison of *in vitro* and *in vivo* patterns could be made between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* representative strains, giving us the real image of the infection capacity of these strains, that normally hide when mixed parasite populations are used. *In vitro* (tested for 4 representative strains) as well as *in vivo* (2 representative strains), it was clear that for the representative strains tested here, *L.* (*V.*) *braziliensis* was more infective than *L.* (*V.*) *peruviana*, showing higher infection indices and faster appearance and larger size of lesions respectively. This correlates with the clinical outcome observed for *L.* (*V.*) *braziliensis*, associated with severe cutaneous and mucosal lesions in the Amazonian basin (Guerra, 1988). Meanwhile, *L.* (*V.*) *peruviana* produces benign cutaneous lesions, never associated with the mucosal phenotype (Lucas *et al.* 1998). Thus our model was sensitive enough to detect phenotypic differences among the representative

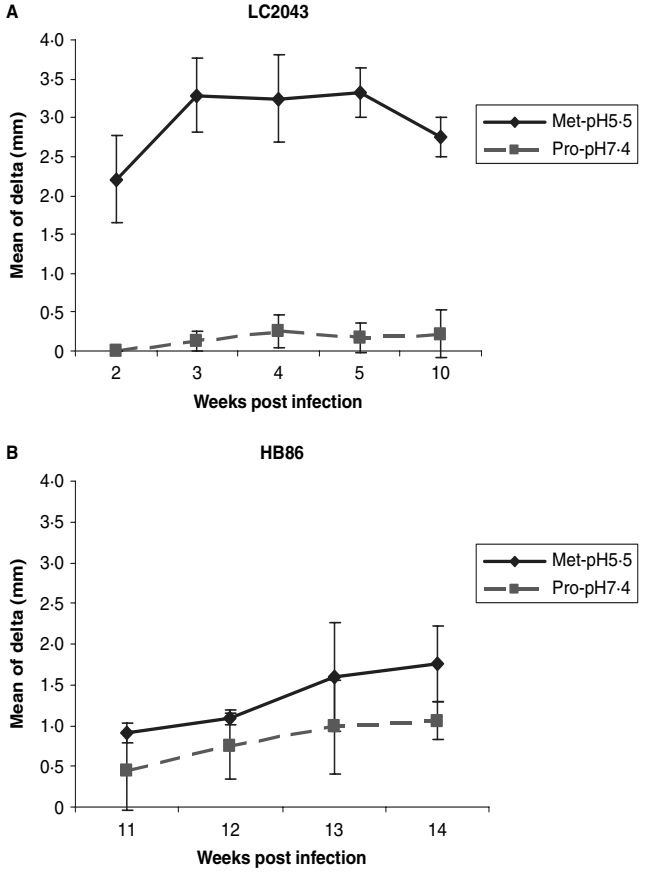

Fig. 2. *In vivo* infections of hamsters using purified metacyclic promastigotes from LC2043 (A) and HB86 (B). Differences were statistically significant from 2 weeks post-infection until the end of the experiment for LC2043 and only at week 14 post-infection for HB86 (ANOVA; $P<0{\cdot}05$, $n=5$).

strains. Our results should be confirmed with a larger sample of the 2 species (from different geographical origin and clinical outcome); but at this preliminary stage, they already converged with different pathogenicities in humans: *L.* (*V.*) *braziliensis*, the most pathogenic in humans, being also more infective in our experimental models. Considering the higher average number of amastigotes observed by *in vitro*-infected macrophages in *L.* (*V.*) *braziliensis*, it would be interesting to measure if this is related to an increased resistance to nitric oxide. This feature could indeed confer a survival benefit for the parasites inside the macrophage and was shown to correlate with disease severity in American tegumentary leishmaniasis (Giudice *et al*. 2007).

A more extensive analysis of the *in vitro* data revealed another interesting result. Indeed, the 4 strains ranked from high to low infectivity as follows: LC2043, LC2177, HB86 and LCA08. It is noteworthy that this corresponded to the gradient of karyotype dissimilarity previously observed among the same strains, which correlated itself with the distance between their geographical origins (Dujardin *et al*. 1993, 1995*b*, 1998). We hypothesized that this dynamic picture would reflect a recent speciation of *L.* (*V.*) *peruviana* from *L.* (*V.*) *braziliensis* (Dujardin *et al*. 1993, 1995*a*, *b*, 1998, 2000, 2002). According to our hypothesis, a *L.* (*V.*) *braziliensis*-like parasite would have colonized the Pacific slopes of the Andes, in the North of Peru (close to the lowest pass across the Andes along the whole Peruvian territory). Then, through a North-South migration and isolation, it would have increased its genomic differentiation. Further work on a larger set of strains is needed to verify this possible correlation between infectivity and geographical location of parasite populations. This may open new perspectives to understand the process of speciation in *Leishmania* and its implications in terms of pathogenicity.

In conclusion, we thus have a model able to demonstrate, in a reproducible way, differences in infectivity among representative strains from *Leishmania* of subgenus *Viannia*. Our results support the role of the parasite in the differences in human pathogenicity observed between *L.* (*V.*) *braziliensis* and

L. (*V.*) *peruviana*. However, further extrapolation should be made with great caution. There is still a huge gap between experimental models and the real situation in humans. *In vitro* models lack the immunological context. On the other hand immunological response may be different between animals and humans and, last but not least, there is a major difference between an infection caused by a needle or by a sandfly bite (immuno-modulatory effect of saliva) (Almeida *et al*. 2003). Further work is thus needed to upgrade *in vitro* as well as *in vivo* models and gather information to extrapolate the data to human infection.

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CHAPTER 5

Putative markers of infective life stages in *Leishmania (Viannia) braziliensis*

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Putative markers of infective life stages in *Leishmania* (*Viannia*) *braziliensis*

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SUMMARY

Gene expression is known to vary significantly during the *Leishmania* life-cycle. Its monitoring might allow identification of molecular changes associated with the infective stages (metacyclics and amastigotes) and contribute to the understanding of the complex host-parasite relationships. So far, very few studies have been done on *Leishmania* (*Viannia*) *braziliensis*, one of the most pathogenic species. Such studies require, first of all, reference molecular markers. In the present work, we applied differential display analysis (DD analysis) in order to identify transcripts that might be (i) candidate markers of metacyclics and intracellular amastigotes of *L.* (*V.*) *braziliensis* or (ii) potential controls, i.e. constitutively expressed. In total, 48 DNA fragments gave reliable sequencing data, 29 of them being potential markers of infective stages and 12 potential controls. Eight sequences could be identified with reported genes. Validation of the results of DD analysis was done for 4 genes (2 differentially expressed and 2 controls) by quantitative real-time PCR. The infective insect stage-specific protein (meta 1) was more expressed in metacyclic-enriched preparations. The oligopeptidase b showed a higher expression in amastigotes. Two genes, glucose-6-phosphate dehydrogenase and a serine/threonine protein kinase, were found to be similarly expressed in the different biological samples.

Key words: *Leishmania* (*Viannia*) *braziliensis*, differential display, gene expression.

INTRODUCTION

Leishmaniasis is still an important tropical disease in the world, affecting people in countries located principally in tropical areas from Latin America, Africa, the Mediterranean basin and Asia. The World Health Organization (WHO) estimates that around 2 million people are infected every year (Desjeux, 2001). The disease is known for exhibiting a wide spectrum of clinical symptoms, ranging from self-healing cutaneous lesions to fatal visceral infections. More than 20 *Leishmania* species have been reported as aetiological agents, and taxonomic diversity is underlying this clinical pleomorphism. For instance, Indian Kala-Azar is caused by *L.* (*L.*) *donovani* and muco-cutaneous leishmaniasis is essentially produced by *L.* (*V.*) *braziliensis*. The variation in clinical manifestation between species is complicated by intra-species variability. For instance, *L.* (*V.*) *braziliensis* will cause a cutaneous lesion with no further progression, but in 10% of the cases, this will be followed by metastasis and severe mucosal mutilations known as Espundia (Llanos-Cuentas, 1993).

The clinical outcome of a *Leishmania* infection results from complex host-parasite relationships. These involve, among others, parasite factors and host factors like the immune status and the genetic background of humans (Liew and O'Donnell, 1993) or the immunomodulatory effect of sandfly saliva (de Almeida *et al*. 2003). Obviously, a multi-disciplinary approach is needed for a comprehensive understanding of this phenomenon.

Identification of the parasite factors involved in virulence (defined by Chang (2003) as the degree of pathogenicity of a microorganism genetically endowed with that capacity, as manifested against a host with an intact immune system under normal conditions) is not an easy task. It requires the understanding of the *Leishmania* life-cycle, with particular attention to the infective stages. During their life-cycle, *Leishmania* spp. pass through 3 major stages, only 2 being infective for vertebrates: the metacyclic promastigotes (the final insect vector stage, programmed for survival in the host and infection of the

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macrophages) and the amastigotes (vertebrate host stage, proliferating in the macrophages).

Gene expression is known to vary significantly during the *Leishmania* life-cycle (Almeida *et al*. 2004) and its monitoring will allow identification of molecular changes associated with the infective stages. Studies on gene expression can be separated in 2 categories, (i) the specific ones, in which expression of a given gene is analysed, and (ii) the random ones in which the expression of global populations of genes is surveyed. Several PCR-based methods have been developed for studies of complex populations of RNAs without knowledge of sequence information (among others, differential display (Liang and Pardee, 1992), subtractive hybridization (Diatchenko *et al*. 1996) and microarrays (Schena *et al*. 1995)). Differential Display analysis (DD) (Liang and Pardee, 1992) provides a fingerprint of cDNA of particular cells and allows the subsequent isolation and further characterization of cDNAs that are potentially of interest. DD analysis was recently used to identify genes preferentially expressed in amastigotes of *L*. (*L*.) *major* (Ouakad *et al*. 2007).

In the present study we focused on *L*. (*V*.) *braziliensis*, a species so far poorly studied in terms of virulence and gene expression, despite being the most pathogenic species in the subgenus *Viannia*. We aimed to identify transcripts that might be (i) candidate markers of metacyclics and intracellular amastigotes of *L*. (*V*.) *braziliensis* or (ii) potential controls, i.e. constitutively expressed; these markers might serve as a reference for future gene expression profiling studies in *L*. (*V*.) *braziliensis*. To that end, we used a parasite strain originating from a patient with a severe mucosal compromise (Espundia), isolated mRNA from procyclic promastigotes, enriched metacyclics and amastigotes and submitted them to DD analysis. After validation by quantitative real-time PCR, we encountered 2 candidate markers of the infective stages and 2 potential controls.

MATERIALS AND METHODS

Parasites

The strain MHOM/PE/91/LC2043 was isolated in Peru from a patient with a severe mucosal compromise. After typing as *L*. (*V*.) *braziliensis* by the isoenzyme analysis (13 enzymes; Bañuls, 1998), the strain was cloned by the micro-drop method (Van Meirvenne *et al*. 1975). Parasites were cultured at 26 °C in a biphasic medium containing base agar (GIBCO) supplemented with 15% defibrinated rabbit blood, overlaid with Locke's solution. Four bottles containing 100 ml of HO-MEM medium supplemented with 10% heat-inactivated fetal bovine serum (56 °C for 30 min), were inoculated with early stationary phase promastigotes at the starting density of 1×10^6 parasites/ml. Parasites were counted daily using a Neubauer brightline haemocytometer.

Promastigotes were grown at pH7·4 and were collected at the logarithmic-growth phase of culture (second day according to the kinetics of the growth curve, further called pH7·4/day2 promastigotes) from 2 of these culture bottles.

An enriched preparation of metacyclic promastigotes was attempted by cultivation of promastigotes at pH 5·5 (Almeida *et al*. 1993; Bates and Tetley, 1993; Zakai *et al*. 1998) and harvesting at the early stationary phase (fourth day of growth according to the growth curve, further called pH5·5/day4 promastigotes). Enrichment in metacyclics was monitored by morphological and biological parameters. The size of the flagellum was measured following the protocol described by Zakai *et al*. (1998), but using an ocular micrometer with 100 rulings corresponding to a spacing of 1·29 μm between rulings in the object plane. The resistance to complement-mediated lysis was measured with the modified protocol from Zakai *et al*. (1998) where we used human serum instead of guinea-pig serum. The *in vitro* infectivity at early stationary phase (day 4, according to the growth curve) was quantified by measuring the percentage of infected macrophages and the average number of amastigotes per infected macrophage.

Amastigotes were obtained from infected Raw 264 macrophages. These cells were infected with pH5·5/day4 promastigotes resuspended in HO-MEM medium supplemented with 10% heat-inactivated fetal bovine serum at 34 °C with a parasite to macrophage ratio of 30 : 1. After 2 h of incubation, the infected cells were washed with the same medium to remove the unattached extracellular parasites and the cultures were incubated for an additional 3 days at 34 °C in 5% CO_2. Amastigotes were released from macrophages after 72 h post-infection by SDS lysis (0·0125%) and further purified by Percoll gradient centrifugation (Hart *et al*. 1981). An additional culture plate, performed under the same conditions described above, was used as a control to monitor the rate of infection.

RNA extraction

For DD analysis total RNA was isolated from 10e7 parasite pellets harvested at the different parasite stages (pH7·4/day2 and pH5·5/day4 promastigotes, and amastigotes) using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Any possible genomic DNA contamination was eliminated by treatment with RNAse-free DNase I (GIBCO). RNA isolated in this manner was resuspended in DEPC-treated water, stored at −70 °C and its integrity was confirmed by formamide gel electrophoresis. For quantitative real-time PCR, RNA was extracted using the RNAqueous kit (Ambion), which includes a DNase I treatment to remove any possible genomic DNA contamination. Quality and quantity of the extracted

Table 1. Oligonucleotide primers used in RNA differential display analysis

*Apo*I-T_{12}CA	5′-TTTTAAATTTTTTTTTTTTCA-3′	21mer
*Apo*I-T_{12}CG	5′-TTTTAAATTTTTTTTTTTTCG-3′	21mer
*Apo*I-T_{12}CC	5′-TTTTAAATTTTTTTTTTTTCC-3′	21mer
*Apo*I-T_{12}CT	5′-TTTTAAATTTTTTTTTTTTCT-3′	21mer
AP-*Eco*RI	5′-GGCGAATTCAGA-3′	12mer
AP-*Bam*HI	5′-GAAGGATCCCAG-3′	12mer
AP-*Hin*dIII	5′-GGCAAGCTTCAG-3′	12mer

RNA was determined using RNA 6000 Nano Labchip kit on the Bioanalyzer 2100 (Agilent Technologies) and the Nanodrop (ND-1000, Isogen-Life Science) respectively.

Differential display analysis

Total mRNA was reversed transcribed with oligo-dT_{12}CX anchor primers (where X = A, C, G, or T), that anneals to the poly(A) tail of all mRNA, in 4 separated cDNA reactions. Theoretically, by using these primers together with 3 random primers one would generate 12 subfractions of cDNA that should represent almost equally one-twelfth mRNA pools. Total mRNA along with 1X first strand buffer (GIBCO), dNTP's (20 μM final concentration), 5 mM dithiothreitol (DTT; GIBCO-BRL), RNAse inhibitor (RNAsin, Promega) and 200 U of Superscript II RNAase H reverse transcriptase (GIBCO-BRL) was added to each tube in a final volume of 20 μl, mixed and incubated for 1 h at 37 °C for cDNA synthesis. Finally the reaction was incubated for 5 min at 94 °C to inactivate the reverse transcriptase. The resulting cDNA (2 μl) was used directly for the radioactive PCR reaction mix (20 μl final volume) containing the respective oligo-dT_{12}CX anchor primer (1 μM final concentration), an arbitrary 12mer oligonucleotide at a final concentration of 0·3 μM, dNTP's, 2·5U Taq polymerase (Eurogentec), 1X Taq reaction buffer (Eurogentec) and 0·2 μl [10 μCi α32P] dCTP (Amersham). The sequences of the primers used in this study are listed in Table 1. PCR cycling conditions for all reactions were 1 cycle at 94 °C for 5 min, 32 cycles of 94 °C 45 s, 38 °C 45 s and 72 °C 1 min 30 s, followed by a final extension at 72 °C for 5 min, and then chilled at 4 °C. The PCR was carried out in a Thermal cycler PTC-100 (MJ Research, Inc.). RNA samples were checked for DNA contaminants by including a reverse transcriptase-free control in all Differential Display PCR experiments (DD-PCR). The total volume of the PCR products was analysed on an 8% denaturing polyacrylamide sequencing gel. After electrophoresis, the gel was carefully removed, transferred to a 3MM Whatman paper and exposed to Amersham Hyperfilm MP overnight at −70 °C. Finally the gel was aligned with the film and the stage-specific bands and some controls were excised with a clean scalpel, eluted and re-amplified in the absence of the labelled nucleotide. To that end, the same primers and reaction conditions were applied as mentioned above, except for an increase in the dNTP concentration from 2 to 20 μM, and differences in the PCR cycling conditions: 30 cycles at 94 °C 30 s, 40 °C 2 min and 72 °C 30 s and a final extension at 72 °C for 5 min. The PCR products were analysed in 2% agarose gel and transferred to Nylon N+ membranes (Amersham) for Southern blotting.

Southern blot analysis

The PCR blots were incubated overnight at 65 °C in hybridization solution (6X SSC, 5X Denhardt's solution, 0·5X SDS) containing genomic DNA of *L.* (*V.*) *braziliensis* (LC2043cl8) that had been randomly ^{32}P-labelled (DecaLabel DNA labelling kit, Fermentas). Filters were washed twice with 2X SSC at 65 °C for 15 min and once with 2X SSC, 0·1% sodium dodecyl sulfate (SDS) at 65 °C for 30 min. After air drying, the filters were exposed to X-ray film at −70 °C.

Cloning and sequencing

All the positive bands after the Southern analysis were re-amplified and purified from the PCR reaction mix using the High pure PCR product purification kit (Boehringer Mannheim) and a restriction reaction was set for each sample according to the specific restriction site of the primers used on the DD-PCR. The purified PCR amplified DNAs were cloned in the pUC19 vector (BioLabs) with T4 ligase (GIBCO-BRL). Sequencing was carried out in a total volume of 20 μl by automated cycle sequencing method on an ABI 3100 DNA sequencer (Perkin Elmer), using universal forward and reverse primers in conjunction with the ABI PRISM Dye Deoxy Terminator Cycle Sequencing kit (PE Applied Biosystems) according to the manufacturer's recommendations.

Bioinformatics analysis

After removal of vector and primer sequences (using Chromas 2.23 software when it was necessary), all the inserts were compared to those in the GenBank

Table 2. Primers used in qRT-PCR

Gene	Forward primer and reverse primer (5′–3′)	Amplicon (bp)	Final concentration in qPCR (nM)
Internal controls			
PAP14	CCTGCTACAATGTTACCCTCACC	116	
	GAACTTCGCCTCCGCCTC		300
S8	CGACTTGGATGCGGGGA	111	
	GGCGAAGCCTTGTTCACG		600
AQP-1	CTTTGCGGTGTGGAGTGAGATA	156	
	CCAGAGTTGATACCTGTCGTGATAC		600
GCS	CTACGACTCTATCTCCATCTTCATCA	115	
	CACACCAGCCTTCTCCAGC		600
Targets			
1CAE (g6pd)	TGTCTGTGGGAGCATTCG	103	400
	GGCGGAACTGTTGGTGTC		
55CAH (stpk)	GTGGTGGCGATGCTGCTA	109	500
	GCCGATGTAGCGAAGTTGG		
29CTH (meta 1)	GGGCAGCGATGACTTGAT	94	400
	CACCAACTTGCCATCCTC		
79CAB (opb)	GAGCACCTCTCGCACATCA	97	300
	GACTGACCTTTCACCTCGC		

sequence database using the BLAST (Basic local alignment search tool) program. BLASTN and BLASTX searchers, from the National Center for Biotechnology Information (NCBI), (http://www.ncbi.nlm.nih.gov), were used for the nucleotide sequences obtained. Generally, hits with BLASTN E values of $<$1e-05 and hits with BLASTX E values of $<$1e-04 were considered significant, although some exceptions were made upon inspection of the alignment. Additionaly GeneDB Data Base (http://www.genedb.org/) for *L.* (*V.*) *braziliensis* and *L.* (*L.*) *major* was also used; we selected the sequences with high Score and low P(N) value for further analysis.

Quantitative real-time PCR analysis

Real-time quantitative PCR (qPCR) using SYBR Green Supermix (Bio-Rad) was used to confirm the differential expression of 4 of the identified clones (further called targets). The cDNA synthesis and qPCR was performed following the protocol described by Decuypere *et al.* (2005*a*), and using an I-cycler (Bio-Rad). The following genes were analysed in parallel with the targets and used as internal expression controls (further called internal controls) for normalization: S8 (internal control used by Coulson *et al.* 1996), PAP14, AQP1, GCS (genes used by Decuypere *et al.* 2005*a*, for *L.* (*L.*) *donovani*); primers used for their amplification and for the amplification of the transcripts selected in the present study are listed in Table 2. All the reactions were done in triplicate using a negative control of cDNA synthesis (i.e. without reverse transcriptase) and non-template controls. The analysis of the quantity data was performed using the geNorm VBA applet for MS Excel developed by Vandesompele *et al.* (2002) and applied by Decuypere *et al.* (2005*a*) for quantification of gene expression in *L.* (*L.*) *donovani*. The raw (non-normalized) expression levels were determined with the delta Ct method, more specifically the Ct value of a gene for a sample was related to the Ct value of the same gene in the sample with the highest expression (or lowest Ct value), taking the amplification efficiency of the PCR for that gene into account. The geNorm VBA applet for MS Excel was used to determine the most stably expressed genes from the set of 8 tested genes (4 targets and 4 internal controls) in a given sample panel and were subsequently used to determine the normalization factor for each sample as described by Vandesompele *et al.* (2002). The relative (normalized) expression levels were obtained by dividing the raw expression levels with the given normalization factor. The 95% confidence intervals (CI) were based on quantitative data collected from 3 repeated experiments that included cDNA-synthesis and quantitative PCR and were calculated on log transformed normalized expression levels, using the T.INV function in MS Excel to calculate the critical t value [CI = mean DPF +/− S.E.M.*t, with S.E.M. = standard error of the mean, and t = critical t value = T.INV (0·05, degrees of freedom)] (Motulsky, 1995).

RESULTS

Features of the biological material used for DD analysis

Procyclic and metacyclic promastigotes are reported to differ in the size of their flagellum, their susceptibility to complement lysis and infectivity *in vitro* and *in vivo* (Bates and Tetley, 1993; Zakai *et al.*

Table 3. Biological features of the parasite material used in DD analysis

Promastigote flagellum size	pH7·4-day2	pH5·5-day4
10–15 μm	27%	12%
20–25 μm	58%	34%
30–35 μm	15%	54%
Percentage of serum producing 50% of promastigote lysis	3·125	6·250
Macrophage infection	**pH7·4-day4**	**pH5·5-day4**
Average number of amastigotes/macrophage	2·33 $\pm$ 0·6	3·6 $\pm$ 0·6

1998). These parameters were measured during the *in vitro* growth of *L.* (*V.*) *braziliensis* LC2043 cl8 in order to monitor the enrichment in metacyclics in pH5·5/day4 promastigotes (Table 3). First, at the morphological level we observed a clear shift of the flagellum distribution size between pH7·4/day-2 and pH5·5/day-4 promastigotes: from a higher percentage around 20–25 μm to 30–35 μm. Secondly, pH5·5/day-4 promastigotes showed a 2-fold increase in their resistance to complement lysis. Thirdly, pH5·5/day-4 promastigotes were found to be significantly ($P<0·05$) more infective to macrophages than pH7·4/day-4 ones: a higher percentage of infected macrophages and a higher average number of amastigotes per macrophage (72 h post-infection). Both preparations obviously remained a mixture of stages (promastigotes at stationary growth phase: procyclics and metacyclics). However, the pH5·5/day-4 promastigotes were clearly more enriched in metacyclics and suited to screen potential markers of this life stage.

Differential display analysis

One of the most critical aspects of DD analysis is the reproducibility during cDNA synthesis and subsequent PCR amplifications. It was necessary to ensure that the observed differences in cDNA pattern between samples from different life stages were due to differences in gene expression and not to variations in the RNA preparation or to PCR artifacts. Therefore, we worked with at least 4 different RNA extractions from different biological batches and tested different RNA concentrations for each sample. Fragments were selected for analysis, only if differential expression was observed in all the repeats of a given life stage. One example of differential cDNA patterns between procyclics, metacyclics and amastigotes is shown in Fig. 1. The product specificity was demonstrated by changing 1 of the random primers, which resulted in a different pattern (data not shown). To verify the efficiency of the DNAse

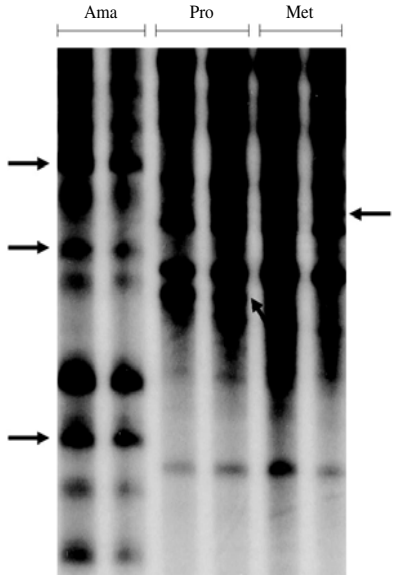

Fig. 1. DD analysis of *Leishmania* (*Viannia*) *braziliensis*: example of stage-specific fragments with primer ApoT12CC-EcoRI (arrows). Ama, amastigotes; Pro, procyclics; Met, metacyclics.

treatment of the RNA, non-reverse transcribed RNA samples from the different life stages were incorporated. As expected, no products were observed following the electrophoresis and autoradiography (data not shown).

Exploitation of the DD analysis

Comparative analysis of the DD patterns revealed that pH7·4/day2 and pH5·5/day4 promastigotes shared about 90% of amplified cDNA fragments mRNAs that were expressed at similar levels. In contrast, our amastigote preparation shared only about 50% of their cDNA fragments with promastigotes. Of course, the latter percentage has to be taken with caution because of the presence of macrophage contaminants (see below).

We isolated a total of 214 DNA fragments (i) 178 differentially expressed between the 3 biological samples (isolated from pH7·4/day2, pH5·5/day4 promastigotes or amastigotes) and (ii) 36 similarly expressed in all the life stages as negative controls. After the hybridization screening with genomic DNA of *L.* (*V.*) *braziliensis* LC2043cl8, 120 fragments were marked as *Leishmania*-specific and selected for further analysis (56%). Of these, 48 DNA fragments gave good sequencing data: 7 isolated from pH7·4/day2 promastigotes, 6 from pH5·5/day4 promastigotes, 23 from amastigotes and 12 controls.

After the analysis of the 48 samples using the GeneDB Data Base for *L.* (*V.*) *braziliensis*, we found only 11 sequences with high Score and low Probability P(N) value (<10e-15), 4 of them

Table 4. Identification of *Leishmania*-specific fragments isolated in *L.* (*V.*) *braziliensis* by DD analysis and showing highest score values

Life stage	Clone code	Accession number	Length	GeneDB *L.* (*V.*) *braziliensis*	Score	Probability P(N)	GeneDB *L. major*	Score	Probability P(N)
All (control)	1CAE	AM420310	357 nt	AM494957 |||glucose-6-phosphate dehydrogenase |Lbraziliensis|chr 20	251	1·5E-24	Q81909 Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	2894	4·00E-303
All (control)	55CAH	AM420311	244 nt	AM494941 |||serine/threonine protein kinase-like protein| Lbraziliensis|chr 4	239	1·40E-19	Q9NLC0 Serine/threonine protein kinase	2566	2·30E-268
Metacyclic	29CTH	AM420313	531 nt	AM494954|Lbraziliensis|chr 17	1798	4·20E-76	Q25307 Infective insect stage-specific protein	336	2·80E-31
Amastigote	79CAB	AM420312	377 nt	AM494946 |||oligopeptidase b|Lbraziliensis|chr 9	423	7·00E-40	AF109875, Q4QHU7 (Lmajor oligopeptidase b)	979	7·70E-118
Amastigote	134CAH	*	437 nt	AM494963 |||p-glycoprotein-like protein|Lbraziliensis|chr 26	667	2·20E-65	Q4Q8S4 P-glycoprotein-like protein (Abc transporter-like protein) (Multidrug resistance protein-like protein)	5315	0
Amastigote	67CAB	*	111	AM494972|||elongation factor 2|Lbraziliensis|chr 35	39	0·11	Q2HZY7 Elongation factor 2	994	8·70E-102
Amastigote	10CAE	*	118	AM494946|Lbraziliensis|chr 9	61	0·074	Q4QHU9 Acyl-CoA binding protein, putative	563	1·70E-54
Amastigote	71CAH	*	140	AM494946|||RNA-binding protein 5-like protein|Lbraziliensis|chr 9	38	0·68	Q4QI26 RNA-binding protein 5-like protein	1328	3·50E-137

* In annotation process.

corresponding to known proteins. When we performed the analysis using GeneDB from *L.* (*L.*) *major*, 40 sequences showed high scores and low P(N) values (<10e-5), from this group only 7 corresponded to known proteins, 3 of them being similar to the ones found for *L.*(*V.*) *braziliensis*. Interestingly, 1 fragment was identified as a portion of the ribosomal RNA gene of *L.* (*V.*) *naiffi* (Rotureau *et al.* 2006). Among the 8 remaining sequences, 5 were identified by BlastN analysis as mouse sequences (all coming from amastigote preparations) and were likely to be contaminants from macrophages. The last 3 sequences did not show significant match. Table 4 presents only the hits corresponding to identified genes, but the complete list is available on request to the authors.

Validation of the gene expression profile by qPCR

Four DNA fragments were selected for qPCR verification of their expression profile in the 3 biological samples (Table 5): they showed a high score and low P(N) value after the GeneDB analysis for *L.* (*V.*) *braziliensis* and/or *L.* (*L.*) *major*, corresponded to known genes and had an appropriate size (more than 200 nt) for primer design for the qPCR analysis. These fragments corresponded to (i) the infective insect stage-specific protein (*meta1*), isolated from pH5·5/day4 promastigotes (thus putative marker of metacyclics), (ii) the oligopeptidase b (*opb*), isolated from amastigotes and (iii) the glucose-6-phosphate dehydrogenase (*g6pd*) and serine/threonine protein kinase (*stpk*), both identified in all life stages. In the case of the *meta1* gene, expression was shown by qPCR to be highest in pH5·5/day4 promastigotes: up to 8 and 12 times higher than in pH7·4/day2 promastigotes (depending on the preparation, which was likely reflecting the proportion of metacyclics) and amastigotes respectively. With respect to the *opd* gene, highest expression was encountered in amastigotes: up to 1·6 and 2·5 times higher than in pH7·4/day2 and pH5·5/day4 promastigotes, respectively. The *g6pd* and *stpk* transcripts showed a similar expression in all samples.

DISCUSSION

In the present work, we applied differential display analysis (DD analysis) for the identification and characterization of mRNA transcripts differentially expressed in different life stages of a *L.* (*V.*) *braziliensis* strain, with particular attention to the infective stages, metacyclics and amastigotes. Fourty-eight fragments gave good sequencing data: among them, (i) 6 were isolated from pH5·5/day4 promastigotes and thus constituted candidate markers of metacyclics and (ii) 23 were candidate markers of amastigotes. Eight sequences only were identified to known genes.

Table 5. Q-RT-PCR normalized expression level of the candidate markers (with standard-deviations) obtained from DD analysis

(Four genes were selected: 2 controls (*g6pd*, glucose-6-phosphate dehydrogenase and *stpk*, a serine/threonine protein kinase) and 2 differentially expressed (*meta1*, candidate marker of metacyclics and *opb*, oligopeptidase b, candidate marker of amastigotes); in bold, higher expression levels observed in pH5·5/day4 promastigotes and amastigotes respectively.)

Life stage	*g6pd*	*stpk*	*meta1*	*opb*
pH7·4/day2 promastigotes	1·07 ± 0·23	0·90 ± 0·08	1·55 ± 0·18	0·61 ± 0·05
pH5·5/day4 promastigotes	1·24 ± 0·08	0·79 ± 0·15	**12·32 ± 1·86**	0·41 ± 0·09
Amastigotes	1·00 ± 0·3	1·00 ± 0·18	1·00 ± 0·47	**1·00 ± 0·11**

DD analysis is one of the methods that can be used for comparing large numbers of mRNA between 2 samples. It has been validated in different organisms and, among others, allowed identification of genes preferentially expressed in amastigotes of *L.* (*L.*) *major* (Ouakad *et al*. 2007) as well as a putative virulence factor (Ben Achour *et al*. 2002). This approach is complementary to other gene expression methodological approaches like suppression subtractive hybridization (SSH, Diatchenko *et al*. 1996) or micro-arrays (Saxena *et al*. 2003; Almeida *et al*. 2004). In our hands, the use of SSH in *Leishmania* was seriously hampered by background caused by the presence of large amounts of poly-adenylated ribosomal RNA (Decuypere *et al*. 2005*b*). This was likely not the problem with DD analysis, as out of 48 sequenced fragments, only 1 corresponded to rRNA. With respect to micro-arrays, this method allowed, for instance, identification of differentially expressed genes in *L.* (*L.*) *major* (Almeida *et al*. 2004; Leifso *et al*. 2007), in *L.* (*L.*) *mexicana* (Holzer *et al*. 2006) and in *L.* (*L.*) *donovani* (Saxena *et al*. 2007). However, this method requires sophisticated equipment and is therefore not as readily available as DD analysis. In addition, micro-arrays currently exist only for *L.* (*L.*) *major*, *L.* (*L.*) *donovani* and *L.* (*L.*) *infantum* and it is likely that a different micro-array would ideally need to be developed for each species.

Whatever the method used for differential expression analysis, the crucial issue is not only the molecular method, but also the biological quality of the material used for mRNA extraction. In the present study, pH5·5/day-4 promastigotes were used as proxy of metacyclics on the base of morphological and functional changes compared with pH7·4/day-2 promastigotes, but we are aware that pH7·4/day-2 and pH5·5/day-4 samples both contain metacyclics, the second one being relatively enriched in this infective stage. This does not disqualify the method used here, nor the candidate markers of metacyclics found using this method, but a qualitatively and quantitatively more precise gene expression profiling would require the use of purified stages. With respect to the amastigotes, our experimental approach was based on *in vitro* intracellular stages and we observed a 50% difference in DD-patterns by comparison with promastigotes. After correction for macrophage contaminants, we estimate that this value should drop by up to 23% (0·5 multiplied by 0·56 – proportion of *L.* (*V.*) *braziliensis* specific sequences as shown by hybridization – multiplied by 0·83 – proportion of *Leishmania*-specific DNA as shown by sequencing). This value is higher than that reported using micro-arrays and axenic amastigotes (3% differential gene, Saxena *et al*. 2007) and the discrepancy could be due to the nature of the biological material. This is supported by micro-array studies that revealed 17 times more differences in gene expression between intracellular amastigotes and promastigotes than between axenics and promastigotes (Holzer *et al*. 2006). However, the values observed in our study are also higher than those observed after micro-array analysis of lesion-derived *L.* (*L.*) *major* amastigotes (1·4%, Leifso *et al*. 2007). We cannot exclude *Leishmania* species-related differences, but these discrepancies also point out the fundamental differences between DD-analysis and micro-array, such as the extent of genome coverage, the sensitivity and the risk with DD-analysis to detect several fragments corresponding to a same transcript. Altogether, particular care should be taken in the interpretation of our DD-analysis results as well as the comparison with other reports made on other species, in biologically different experimental conditions and with other molecular methods.

With regard to the high frequency of false positive results with DD analysis, confirmation of the expression profiles needed to be performed with a more robust technique for relative quantification of transcripts. This was done here by quantitative reverse transcription PCR, for 4 candidate markers: 2 differentially expressed in DD analysis and 2 controls. (1) The transcripts corresponding to the infective-insect stage protein (also known as the Meta 1 protein) were identified as candidate markers of metacylics. This gene was first identified by Coulson and Smith (1990) in *L.* (*L.*) *major*, and then in *L.* (*L.*) *donovani* and *L.* (*L.*) *amazonensis* by Uliana *et al*. (1999). This gene is predominantly expressed in infective metacyclics of *L.* (*L.*) *major* (Nourbakhsh *et al*. 1996) and over-expression of corresponding Meta 1 protein in *L.* (*L.*) *amazonensis* generates parasites that are more virulent than wild-type organisms *in vivo* (Uliana *et al*. 1999). Our results support the hypothesis that

the product of this gene could be an important virulence factor in parasites of the subgenus *Viannia*. (2) The transcripts corresponding to the oligopeptidase B were identified as candidate markers of amastigotes. This is a serine oligopeptidase, a member of the prolyl oligopeptidase family (Morty *et al*. 1999), involved in (i) Ca(2+)-signalling during host cell invasion by *Trypanosoma cruzi* (Burleigh *et al*. 1997; Caler *et al*. 1998) and (ii) in degradation of regulatory peptide hormones in the blood of infected hosts in African tryponosomiasis (Troeber *et al*. 1996). So far, it has not been reported as a potential virulence factor in leishmania. (3) Transcripts corresponding to a serine/threonine protein kinase were likely expressed at the same level in the different biological preparations. Protein kinases constitute a large family of important regulators of many different cellular processes such as transcriptional control, cell cycle progression and differentiation (Naula *et al*. 2005). A large number of protein kinases (179) were identified in *Leishmania* (Naula *et al*. 2005), some of them being exclusively expressed in a given stage (f.i. LmxMKK in the promastigote stage, Wiese *et al*. 2003). For others (like CRK3), mRNA was found to be constitutively expressed throughout the parasite life-cycle (Wang *et al*. 1998). (4) The transcripts corresponding to the glucose-6-phosphate dehydrogenase were also found to be constitutively expressed in the strain here analysed. A proteomic study of *L*. (*V*.) *panamensis* revealed that this protein was more abundant in amastigotes (Walker *et al*. 2006). This protein could be another example of post-transcriptional regulation mechanisms which are common in *Leishmania* and other Trypanosomatids (Stiles *et al*. 1999). Noteworthy, this is likely not the case for all genes of the parasite: there was for instance 53% correlation between *L*. *infantum* amastigote-specific protein isoforms and mRNA expression trends (McNicoll *et al*. 2006). In a screening phase, transcript levels would be enough to identify potential markers, but particular care should be taken when interpreting their biological meaning and this should be completed by functional studies, including analyses at the protein level.

The present study demonstrates the feasibility of DD screening for transcripts coming from parasites obtained at different life stages and provided a set of potential markers of the infective stages of *L*. (*V*.) *braziliensis* that might be used as references when monitoring gene expression in that species. Further work is needed to validate our markers in other strains of that species and in related less-pathogenic species like *L*. (*V*.) *peruviana*. Last but not least, it should be tested whether some of them are indeed virulence factors in *L*. (*V*.) *braziliensis*.

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CHAPTER 6

Gene expression analysis during *L.braziliensis* and *L. peruviana in vitro* life cycle: a pilot study

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(manuscript in preparation)

SUMMARY

L. (V.) braziliensis and *L. (V.) peruviana* are two very closely related species at genomic level; but with different clinical outcomes and geographical distribution. There are few studies trying to elucidate the parasite factors that could be responsible of the differences in pathogenicity between these two species. In order to explore this we followed, by Quantitative PCR (qPCR), the expression of two putative markers of infective stages (*'meta 1'* and *'opb'*) throughout the *in vitro* life cycle of 4 strains (two representative strains of *L. (V.) braziliensis* from cutaneous and mucosal origin respectively and two representative strains of *L. (V.) peruviana*). In the case of *'meta 1'* gene, we found an up-regulation of expression at stationary stage (day 4 and day 5) in the four strains used here and the *'opb'* was found to be over-expressed in amastigote stages of *L. (V.) braziliensis* strain LC2177, while no signal was detected at this stage in *L. (V.) peruviana.*

INTRODUCTION

L. (V.) braziliensis and *L. (V.) peruviana* are causing a very different pathology in humans despite a high genetic similarity. *L. (V.) braziliensis* produces cutaneous lesions leading to metastasis (mucosal lesion called 'espundia') in approximately 10% of the cases (Llanos-Cuentas, 1991) and is mainly present in the Amazonian forest. *L. (V.) peruviana* is prevalent in the western Andean and inter-Andean valleys and produces mild cutaneous lesions (called 'uta') (Herrer 1962, Lumbreras and Guerra 1985, Guerra 1988). Even if host factors might play a role in this clinical pleomorphism, parasite factors are likely involved. Indeed, we recently showed *in vitro* as well as *in vivo* that *L. (V.) braziliensis* was more infective than *L. (V.) peruviana* (Gamboa et al., 2007a). This opened new perspectives for the understanding of the high pathogenicity of *L. (V.) braziliensis.*

During the Leishmania life cycle, in the mammal host and in the sandfly vector, the parasite undergoes morphological and biological changes, which include the promastigote stage with variable morphology located inside the gut of the female sand fly (long flagellated parasite), and the amastigote stage (round form without free flagellum) inside the phagolysosome of the mammal host. All these changes in the expression of specific genes will allow the parasite to survive in these two very different environments. The more interesting parasite forms in terms of virulence and pathogenicity are the metacyclic promastigotes (the final insect vector stage, programmed to infect the mammalian host), responsible of initiating the infection, and the amastigotes forms (vertebrate host stage, proliferating inside the macrophages), which maintain the infection in the mammalian host.

Expression of a number of genes has been reported to vary significantly during the Leishmania life cycle (Almeida et al., 2004, Holtzer et al*.,* 2006, Cohen-Freue et al., 2007, Ouakad et al., 2007, among others) and its monitoring allows identification of molecular changes associated with the infective stages. In a previous study we identified in a reference strain of *L. (V.) braziliensis* two genes that were over-expressed in the metacyclic (infective insect stage-specific protein *'meta1'*) and amastigote (oligopeptidase b *'opb'*) stages respectively (Gamboa et al 2007b). Monitoring these putative markers of infective stages in *L. (V.) braziliensis* and *L. (V.) peruviana* would contribute to the understanding of the differences in pathogenicity between these two species.

A first exploration was done with two representative strains of *L. (V.) braziliensis* from cutaneous and mucosal origin respectively and two representative strains of *L. (V.) peruviana.* The *'meta1'* and *'opb'* genes were followed during the consecutive stages of the parasite life cycle with particular attention to the infective stages. For the *'meta1'* gene, we found an increased relative expression level at stationary ("infective") stage (day 4 and day 5) in the four strains used here. The *'opb'* was found to be upregulated in amastigote stages of *L. (V.) braziliensis* while no signal was detected in *L. (V.) peruviana.*

MATERIALS AND METHODS

Leishmania *strains*

The *L. (V.) braziliensis* strains used in present study originated from the same locality in the Amazonian basin: MHOM/PE/91/LC2043 (mucosal origin), and MHOM/PE/91/LC2177 (cutaneous origin). The two *L. (V.) peruviana* strains were both of cutaneous origin but from different localities: MHOM/PE/90/HB86 (Andes from the North) and MHOM/PE/90/LCA08 (Andes from the South). The strains were cloned by the micro-drop method (Van Meirvenne et al. 1975) and characterized by multi-locus enzyme electrophoresis (13 enzymes, Bañuls 1998). Identity of the strains was confirmed by Pulsed Field Gradient Electrophoresis before the beginning of the experiments, as described elsewhere (Dujardin et al. 1987). For each strain, cryostabilates made from parasites with a minimum number of subinoculations were thawed and first cultivated at 26°C in a biphasic agar medium supplemented with 15% defribinated rabbit blood and 0.85% saline solution. Then, they were adapted to grow at 26°C in an enriched medium, M199 (SIGMA®), supplemented with 20% (v/v) heat inactivated Fetal Calf Serum (FCS) and adjusted at pH 7.4. In order to have enough parasites for the experiments, promastigotes at early stationary phase ($1x10^6$ parasites/ml) were sub cultivated into one bottle with 50ml of medium M199 at pH7.4, and was incubated at 26°C. The growth curve was monitored by daily counting of parasites using a Neubauer brightline haemocytometer (from day 1, to day 8) and harvested for further RNA isolation.

Intracellular amastigotes: in vitro generation and purification

Amastigotes were obtained from infected Raw 264 macrophages cells. These cells were infected with promastigotes at stationary phase (pH7.4 day 5) resuspended in HO-MEM medium supplemented with 10% heat-inactivated fetal bovine serum at 34°C with a parasite to macrophage ratio of 30:1. After 2 h of incubation, the infected cells were washed with the same medium to remove the unattached extra-cellular parasites and the cultures were incubated for additional 3 days at 34°C in 5% CO2. Amastigotes were released from macrophages after 72 h post-infection by SDS lysis (0.0125%) and further purified by Percoll gradient centrifugation (Hart et al., 1981). An additional culture plate, performed under the same conditions described above, was used as control to monitoring the rate of infection.

RNA isolation and analysis

All parasites, cultured and harvested as described above, were immediately frozen at -80°C until RNA extraction was performed. Total RNA was extracted using the RNAqueous kit (Ambion), and all samples were DNAse treated using the DNA-free kit (Ambion) to remove possible contaminating genomic DNA. Quality and quantity of the resulting RNA were determined using the RNA 6000 Nano Labchip kit on the Bioanalyzer 2100 (Agilent Technologies).

cDNA synthesis and real-time quantitative PCR

Total RNA (~150 ng/reaction) was reverse transcribed at 55°C with Transcriptor Reverse Transcriptase (ROCHE®) using conditions recommended by the manufacturer with a 15mer oligo(dT). The resulting cDNA was diluted 10 times, and 2µl was added to 25µl quantitative PCRs for expression profiling of two genes identified as putative markers of infective life stages in *L. (V.) braziliensis*, and of two genes identified as potential controls included here for normalization purposes, referred to as internal controls (Table 1). Quantitative PCR, contained 1X iQ Sybr Green Supermix (Bio-Rad®) and forward and reverse primers as specified in Table 1, was performed. Reactions were run on an iCycler (Bio-Rad®) using the following thermal profile: initial denaturation at 95°C for 5 min followed by 34 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. The PCR was immediately followed by a melt curve analysis using temperature increments of 0.5°C every 30 s to ascertain if the expected product was amplified and to ensure no non-specific products or primer dimers (which could bias the quantification) were formed. The following controls were included in each run for each gene: (i) two serial dilution points of promastigotes sample from each specie (*L. (V.) braziliensis*: LC2043; *L. (V.) peruviana*: LCA08) to ascertain consistent PCR efficiency, (ii) negative controls of cDNA synthesis (i.e., without reverse transcriptase) "NRT controls", and (iii) no-template controls. All reactions were done in triplicate, with their arithmetic average threshold cycle (CT) used for data analysis.

Table 1: Target genes and internal controls

GENE	PROTEIN	FUNCTION	Forward and Reverse primer (5' – 3')	Final concentration in qPCR (nM)
'meta1'	Infective insect stage-specific protein (Meta 1 protein)	Putative marker of metacyclic	GGGCAGCGATGACTTGAT CACCAACTTGCCATCCTC	400
'opb'	Oligopeptidase b	Putative marker of amastigotes	GAGCACCTCTCGCACATCA GACTGACCTTTCACCTCGC	300
'g6p'	Glucose-6-phosphate dehydrogenase	Internal control	TGTCTGTGGGAGCATTCG GGCGGAACTGTTGGTGTC	400
'stpk'	Serine/threonine protein kinase-like protein	Internal control	GTGGTGGCGATGCTGCTA GCCGATGTAGCGAAGTTGG	500

Analysis of quantitative data and applied statistics

The raw (non-normalized) expression levels were determined with the delta CT method, more specifically the CT value of a gene for a sample was related to the CT value of the same gene in the sample with the highest expression (or lowest CT value), taking the

amplification efficiency of the PCR for that gene into account. The geNorm VBA applet for MS Excel was used to determine the most stable expressed genes from the total set of 4 tested genes (data not shown) in a given sample panel and were subsequently used to determine the normalization factor for each sample as described by Vandesompele et al. (2002). The relative (normalized) expression levels were obtained by dividing the raw expression levels by the given normalization factor.
The whole experiment, from RNA extraction, cDNA synthesis and quantitative PCR, was done in triplicate (in two different laboratories) in order to confirm the results obtained.
The statistical analysis was done with GraphPad Prism® for Windows, from GraphPad Software, Inc. A non-parametric Mann Whitney test (MW test) was used to compare various groups of expression level data determined at different time-points of the growth curves. In addition, the 95%CI of the expression level at each data-point were determined to enable comparison of the individual data points between the four strains.

RESULTS AND DISCUSSION

Having potential markers of infective stages in *L. (V.) braziliensis* (Gamboa et al., 2007b), our aim in the present pilot study was to monitor these putative markers during the *in vitro* Leishmania life cycle using 2 representative strains of *L. (V.) braziliensis* (from cutaneous and mucosal origin) and 2 representative strains of *L. (V.) peruviana* (cutaneous origin) for a further exploration of the differences in pathogenicity between these two species. To that end we used Quantitative Real time PCR and the normalization process described by Vandesompele et al. (2002).

Leishmania promastigotes growth curves and in vitro infections

At this first step, we monitored the growth curve of the four species by daily counting and we found that the four Leishmania strains showed a similar growth curve during their *in vitro* culture during 8 days (Fig. 1). The logarithmic stage presented a similar slope in all the strains (from day 1 to day 3). The parasites reached the stationary phase at day 5 in all strains; but in the strain LCA08, it reaches at day 4. From day 6 until the end all the strains displayed a similar decrease in parasite density. Each growth curve represents the average of three growth curves made in parallel for each strain. The small standard deviation amongst the three independent samples indicates a good reproducibility of our *in vitro* system. Five time points were selected for the gene expression analysis of the promastigote life stage: (i) logarithmic (proliferative stage, day 2), (ii) early stationary stage (day 4), (iii) promastigote at stationary stage (day 5) and (iv) late stationary stage (day 6 and day 8).
With respect to the amastigotes, our experimental approach was based on *in vitro* intracellular stages. To obtain this, we used day-5 promastigotes to infect the macrophages, and 72 hours after the infection we determined the percentage of infected cells and the results ranked as following: 75% (LC2043cl8), 65% (LC2177cl 2), 62% (HB86cl4) and 60%

(LCA08cl2). The amastigotes parasites harvested after 72 hours post infection at pH 7.4 were used for the further gene expression analysis (considered as day 11 in the gene expression curves, see hereafter).

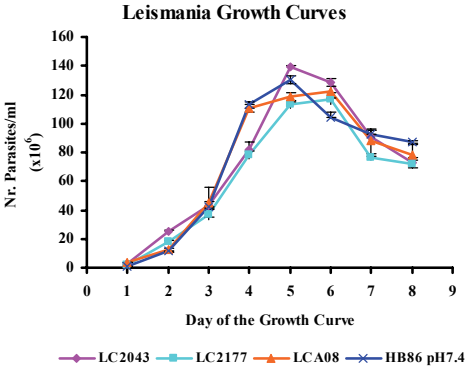

Fig. 1 *Growth curves of L. (V.) braziliensis and L. (V.) peruviana strains in HO-MEM medium at pH7.4. Each point on the curve represents the average of three independent growth curves (X±SD)*

Gene expression profiling during the in vitro *Leishmania* life cycle

The expression of two putative markers of infectivity and two controls was analysed during the *in vitro* life cycle of *L. (V.) braziliensis* (highly pathogenic species) and *L. (V.) peruviana* (low pathogenic). The reliability of the generated data depends largely on the quality of the biological material used for mRNA extraction. As a first approach, in this preliminary study we used promastigotes that were kept at pH 7.4 along the growth curve, being aware that these samples contain mix of parasites populations (procyclics and metacyclics). This does not disqualify the method used here because afterwards we used enriched metacyclics preparation (growth at pH 5.5) to confirm the preliminary results. Additionally, we used intracellular amastigotes instead of axenic amastigotes, the latter has been shown to have only 3% differential gene compared with promastigotes (Saxena et al., 2007).

Quantitative PCR assays were designed and individually optimized for the four genes in order to obtain robust and reliable quantification assays. All PCRs had efficiency above 80% and also the melt curve analysis after the RT-PCR showed the amplification of the expected size product and no primer dimers. The primers were previously shown to amplify genomic DNA from the four representative strains (data not shown). Of the four genes here analysed, expression of *'g6p'* and *'stpk'* was the most stable along the *in vitro* Leishmania life cycle for the four strains used here (Fig. 2a and 2b), hereby confirming previous findings (Gamboa et al., 2007b).

In the case of *'meta 1'* gene, we found an increased relative expression level (three to eleven folds; MW test P value 0.0273) at the stationary ("infective") stage (day 4 and day 5) in the four strains used here, which corresponds with the start of the stationary phase of

the growth curve (Fig. 3). The strain LCA08 had the highest relative expression level at day 4 (nine folds increase) comparing with the other three strains, which could be explained by an earlier maturation of that strain under our experimental conditions, as suggested by the growth curve (Fig. 1). It is known that at the start of the stationary phase, the proportion of metacyclics is higher, and indeed the *in vitro* infections of macrophages initiated with these specific parasites were successful (data not shown). In addition, there were also differences in the height of the *'meta 1'* peaks among the strains, with a significant higher expression in the strain LC2043, suggesting different proportions of metacyclics in the 4 samples.We also found that the expression of *'meta 1'* in an enriched metacyclic preparation obtained from a culture at pH5.5 was higher than the non-enriched sample at the same day of growth (Day 5) for all the strains studied except for LC2177 (Fig. 4). This indicates that the experimental procedure indeed allowed enrichment in metacyclics, except for LC2177.

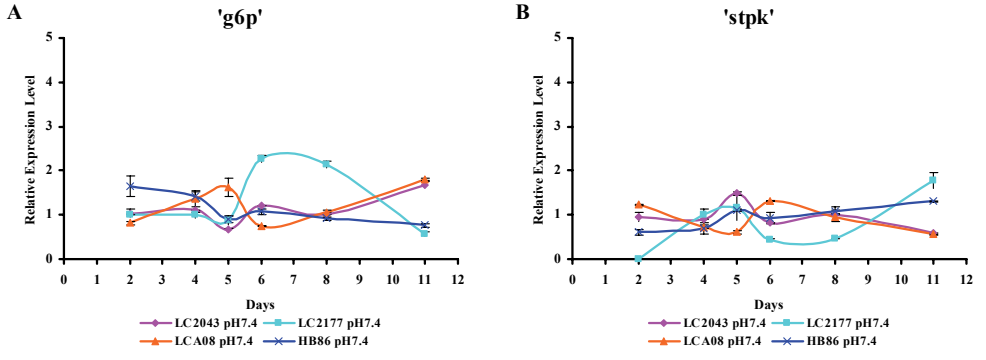

Fig. 2A and 2B *Gene expression profiling of internal control genes ('g6p' and 'stpk') throughout the Leishmania in vitro life cycle.*

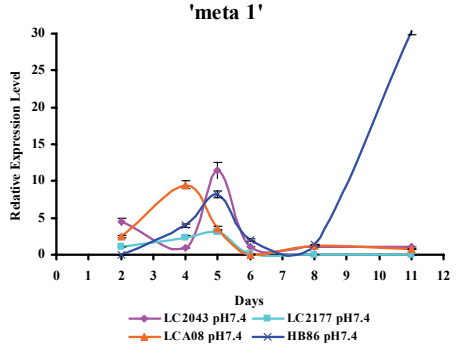

Fig. 3 *Gene expression profiling of 'meta 1' throughout the Leishmania in vitro life cycle (promastigote stage: day 2, 4, 5, 6, 8 and amastigote stage: day 11).*

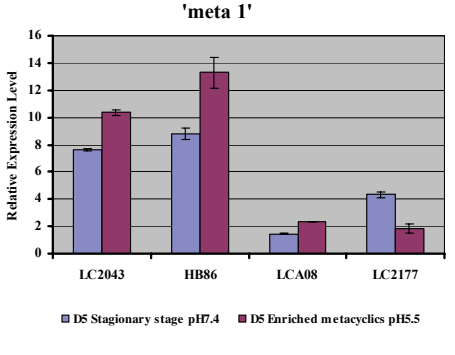

Fig. 4 *Gene expression of 'meta 1' at pH5.5 comparing non-purify metacyclics (D5 stationary stage) versus purify metacyclics at Day 5*

The *'meta 1'* gene is expressed in Leishmania insect stages in particulary in metacyclics which are highly infective, complement-resistant and very well adapted to infect the host (Sacks and Perkins 1984, Tetley and Bates 1993). This gene was originally found in a cDNA library screening (Coulson and Smith 1990) and has been extensively studied at genomic level in *L. major* (Nourbakhsh 1996) and in other especies like *L. donovani* and *L. amazonensis* (Uliana 1999), *L. infantum, L. braziliensis* and *L. panamensis* (Berberich et al 1998). Over-expression of this gene in a mutant parasite line resulted in an increased infectivity during *in vivo* infection experiments in susceptible and partially resistance strains of mice which are indicative for increased virulence of this particular mutant (Uliana 1999). So, our findings for the *L. (V.) braziliensis* and *L. (V.) peruviana* strains appear to largely in line with this previous performed work and the hypothesis that this metacyclic-specific protein could be involved in mechanisms directed to disable the macrophage to kill the invading organisms. The observation of a second peak of expression of *'meta 1'* in the amastigote stage of HB86 is difficult to explain. In line with Uliana and coworkers (1999) who reported that *'meta 1'* transcription was barely detected in amastigotes, we did not find any significant expression of *'meta 1'* in amastigote stages of the other strains. One explanation could be that in HB86, the washing of the infected macrophages (made to remove the unattached extra-cellular parasites) could have been of low efficiency, and that extracellular parasites (metacyclics) would have remained together with the infected macrophages.

In the case of amastigote putative marker *'opb'*, gene expression was variable in the promastigote stages in the four strains analyzed here (Fig. 5), reaching the highest level at day 6-8 for the *L. (V.) braziliensis* strain LC2177. At amastigote stage, *'opb'* was found to be up-regulated in amastigote stages of *L. (V.) braziliensis*, strain LC2177 (3-fold increase compared with promastigotes) as previously reported for another strain of that species, LC2043 (Gamboa et al., 2007b), and no signal was detected in *L. (V.) peruviana*

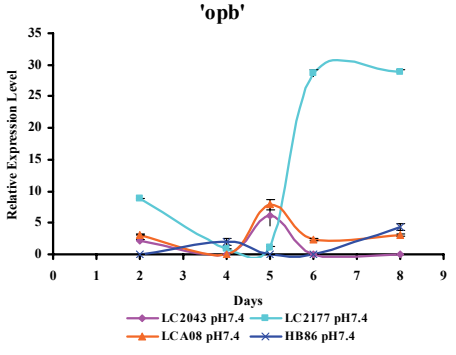

Fig. 5 *Gene expression profiling of 'opb' during Leishmania in vitro promastigoet growth curve (promastigote stage: day 2, 4, 5, 6, 8).*

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Altogether, these results suggest a lower gene expression of *'opb'* in *L. (V.) peruviana*, in comparison to *L. (V.) braziliensis* under our experimental conditions and using very strict parameters for the quantification/normalization process described by Vandesompele et al., (2002).

Oligopeptidase B *('opb'*) was previously identified in *Trypanosome brucei* as new member of the Prolyl Oligopeptidase family of Serine Hydrolases and share 70% amino acid sequence with the intracellular pathogen *Trypanosome cruzi* where it has shown to have an important role in the mammalian host cell signalling and invasion (Burleigh et al 1997 and Caler et al 1998, Morty et al 1999,). The presence of serine proteases in Leishmania has been proved in previous studies (Colmenares et al 2001, Silve-Lopez and De Simone 2004, Silva-Lopez et al 2005, Guedes et al 2007) and the presence of serine oligopeptidades was reported by Ribeiro de Andrade et al 1998. More recently Guedes et al (2007) amplified the complete open reading frame of oligopeptidase B from *L. (L.) amazonensis* using primers designed for the oligopeptidase B gene from *L. (L.) major*. It codifies for a protein of 731 amino acids and shares a 90% identity with oligopeptidases of *L. (L.) major* and *L. (L.) infantum*, 84% with *L. (V.) braziliensis*, and approximately 62% identity with *Trypanosoma* peptidases. Even tough Guedes et al (2007) reported the expression of this gene throughout the life cycle of the parasite, it is important to state the fact that for their RT-PCR (reverse transcription) assay, promastigotes (at logarithmic and stationary stage) and axenic amastigotes were used, while we used here intracellular amastigotes and quantitative PCR that has showed to be a reliable and sensitive technique for the quantification of mRNA (Ding and Cantor 2004) and the normalization process with internal control genes described by Vandesompele et al (2002).

In conclusion, our two markers open interesting avenues for a better understanding of the pathogenicity of *L. (V.) braziliensis* and *L. (V.) peruviana*. Further strains should be analysed in order to check the level of *'meta 1'* expression in metacyclics, and this gene can be used for monitoring metacyclogenesis (for instance to optimalise *in vitro* and *in vivo* infections). Similarly, our findings in the *'opb'* differential gene expression should be expanded, (i) to verify if this could indeed be a marker for the higher pathogenicity of *L. (V.) braziliensis* (in comparison to *L. (V.) peruviana*) and (ii) to check if *'opb'* gene expression within *L. (V.) braziliensis* strains could be related to the different clinical outcomes described for these species (mucosal and cutaneous disease). In addition, further functional assays with the proteins (Meta 1 and Oligopeptidase b) should be also performed in order to understand their possible role as virulence factors in Leishmania.

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CHAPTER 7

GENERAL DISCUSSION

New World leishmaniasis in South America is mainly caused by *L. (V.) braziliensis and L. (V.) peruviana*, two closely related species on a genetic level, but with different clinical outcomes. *L. (V.) braziliensis* produce cutaneous lesions leading to metastasis (mucosal lesions called 'espundia') in approximately 10% of the cases (Llanos-Cuentas, 1991) and is mainly present in the Amazonian forest. *L. (V.) peruviana* is prevalent in the Western Andean and inter-Andean valleys and produces mild cutaneous lesions (called 'uta') (Herrer 1962, Lumbreras and Guerra 1985, Guerra 1988).

The main objective of this thesis was to explore the role of the parasite in terms of differences in pathology between *L. (V.) braziliensis* and *L. (V.) peruviana*, and to identify parasite factors involved in Leishmania pathology. In **Chapter 2**, we highlighted a number of genomic differences between the two species, which confirm that we were indeed dealing with two distinct groups of parasites, and we elaborated on the evolutionary relationships between them. We used chromosome size polymorphism and appropriate algorithms for data processing in order to test an evolutionary hypothesis about the origin and evolution of these two species. Of the 35 chromosomes in subgenus *Viannia* (Britto et al. 1998), five with significant size polymorphism were selected for the analysis, using the absolute chromosome size difference index (aCSDI) method developed by Dujardin et al. (1995). We used isolates of *L. (V.) braziliensis* and *L. (V.) peruviana* from different biogeographical units (BGUs) described in Peru on the basis of the butterfly species distribution (Lamas 1982). All *L. (V.) braziliensis* isolates could be grouped in small clusters at distance from *L. (V.) peruviana* isolates. The latter displayed a much higher chromosome size polymorphism and its populations were structured according to their BGU of origin along a north-south cline. *L. (V.) peruviana* isolates from the north showed a higher karyotype similarity with *L. (V.) braziliensis* than with *L. (V.) peruviana* from the south. These northern *L. (V.) peruviana* isolates were collected in the BGU close to the Porculla Pass, which is the only natural pass in the Peruvian Andes that connects the Amazonian forest with the Pacific slopes. The calculated divergence time of *L. (V.) peruviana* and *L. (V.) braziliensis* is less than 1.5myrs. Therefore it is likely that both species diverged after the Andes mountain chain was formed (estimated 3myrs ago, Van der Hammen 1982). Accordingly, we think that the picture of karyotype variations observed reflects the evolution of *L. (V.) peruviana* from *L. (V.) braziliensis* and the course of its colonization from the Porculla Pass over the Pacific slopes of the Andes. During its North-South migration, passing through various Pacific BGUs and their respective sandfly species, *L. (V.) peruviana* appears to have increased its genetic and genomic diversity (Davies et al. 1993, Villaseca et al. 1993, Caceres et al. 2004).

Parallel studies have proven that the chromosome size polymorphism described above was related to significant rearrangements in chromosomes carrying genes important for parasite virulence, such as gp63 (Victoir et al. 1995) and cystein proteinase b (Polet 1999). These studies support the contribution of parasite factors to the difference in pathogenicity observed in humans. However, such molecular markers might be confounding factors and further evidence of the role of both parasite and host should come from

experimental *in vitro* and *in vivo* studies. Such studies have been mainly conducted for Leishmania strains of the subgenus Leishmania*, e.g. L. (L.) major*) (Garin et al. 2001, Achour et al. 2002, Baldwin et al. 2003, Vladimirov et al. 2003, Sádlová et al. 2006).

The possibilities to employ molecular DNA based techniques to detect and characterize L.(V.) peruviana in the vector were tested on a number of Lutzemia peruensis samples collected in the field, as described in **chapter 3**. Samples were inoculated in hamsters. In addition, the samples were subjected to PCR followed by restriction analysis. The results show a relatively low grade of infection of the sandflies. The PCR-based method appeared to be superior to other techniques to analyze Leishmania infections in the vector, such as inoculation in live animals. It is rapid and reliable. Because this technique is versatile and sensitive it may be useful in future studies into the identity and characteristics of Leishmania parasites in the vector. Also, it will be valuable to perform large epidemiologic studies, since it is less time-consuming, and it may be used to monitor the changes in the DNA that lead to differences in pathology.

In order to gain insight into the differences in virulence between both species, in **Chapter 4** we addressed the biological differences between representative strains of *L. (V.) braziliensis and L. (V.) peruviana*. We developed an *in vitro* and *in vivo* model for experimental infection using purified metacyclics to infect macrophage cell lines and golden hamsters. To this end, we selected two representative strains of *L. (V.) braziliensis,* from cutaneous and mucosal origin, and two representative strains of *L. (V.) peruviana,* from northern and southern Peru respectively. The metacyclic stages of the Leishmania parasite have biological features that allow the parasite to infect the host with great efficacy, though this infective capacity differs among strains. To obtain a proper view on virulence it was essential to work with Leishmania populations that are as homogeneous as possible. Therefore, infective-stage parasites (metacyclics) were enriched in culture and purified to be used as sources of infection with the aim of providing reliable data on the infection capacity of these strains, which is normally underestimated because of the use of mixed parasite populations. We showed *in vitro* as well as *in vivo* that under our experimental conditions, the *L. (V.) braziliensis* isolates were more infective than the *L. (V.) peruviana*. This is in line with the clinical outcome of these infections: *L. (V.) braziliensis* is associated with severe cutaneous and mucosal lesions (Guerra 1988), while *L. (V.) peruviana* produces benign cutaneous lesions and is never associated with the mucosal phenotype (Lucas et al. 1998). Extensive analysis of the *in vitro* data revealed that variations in infectivity of the tested strains correspond with degrees of karyotype dissimilarity previously observed among those strains (Chapter 2) as well as with the distances between their geographical origins (Dujardin et al. 1993, 1995b, 1998). Our results point to a role of the parasite in the differences in human pathology observed between *L. (V.) braziliensis* and *L. (V.) peruviana* infections, calling for a further search for parasite markers that could be related to these differences in pathology. We decided to use a strategy based on a search focusing on differences at the transcriptional level rather than on variations in the parasites' genomic make-up.

In Chapter 5 we first aimed to identify transcriptomic markers of the infective stages (metacyclics and amastigotes) of one representative strain of *L. (V.) braziliensis*. Rather than focusing on specific genes (an approach explored in a parallel PhD study), we analyzed global populations of mRNA, using the Differential Display (DD) technique. This technique has been applied for several organisms and, among other things, enabled us to select genes preferentially expressed in amastigotes of *L. (L.) major* (Ouakad et al. 2007) as well as identifiying a putative virulence factor (Ben Achour et al. 2002). This approach is complementary to other gene expression analysis approaches like suppression subtractive hybridization (SSH) (Diatchenko et al. 1996) or microarrays (Saxena et al. 2003; Almeida et al. 2004). In a previous estudy, the use of SSH in Leishmania was seriously hampered by the presence of large amounts of polyadenylated ribosomal RNA (Decuypere et al. 2005). This was not a problem in DD analysis, where only one out of 48 sequenced fragments corresponded to rRNA. The microarrays method allowed the identification of differentially expressed genes in *L. (L.) major* (Leifso et al. 2007; Almeida et al. 2004), *L. (L.) mexicana* (Holzer et al. 2006) and *L. (L.) donovani* (Saxena et al. 2007). However, this method requires sophisticated equipment, is costly and is therefore not as readily available as DD analysis. Besides, microarrays currently exist only for *L. (L.) major, L. (L.) donovani* and *L. (L.) infantum*. The reliability of the data generated depends largely on the quality of the biological material used for mRNA extraction. In this study we used pH5.5/day-4 promastigotes as proxy of metacyclics on the basis of morphological and functional changes compared with pH7.4/day-2 promastigotes; both samples contain metacyclics. The first sample is enriched for the infective metacyclic stage. This does not disqualify the method used and the candidate markers of metacyclics found. However, a qualitatively and quantitatively more precise gene expression profiling will require the use of better purified stages. With respect to the amastigotes, our experimental approach was based on *in vitro* intracellular stages, and after correction for macrophage contaminants we observed a 23% difference in DD patterns by comparison with promastigotes. This value is higher than that reported when using microarrays and axenic amastigotes (3% differential gene, Saxena et al. 2007), a discrepancy that might be due to the nature of the biological material. Using DD, 48 DNA fragments gave reliable sequencing data, 29 of them being potential markers of infective stages and 12 potential controls. Eight sequences have been previously reported. Thus, the majority of the genes obtained were unknown. This directly shows one of the strengths of the presented approach: selection of 'new' genes involved in Leishmania pathology.

Validation of the results of DD analysis by quantitative real-time PCR was done for four genes (two differentially expressed and two controls). The infective insect stage-specific protein (*'meta 1'*) was more expressed in metacyclic-enriched preparations. The oligopeptidase b (*'opb'*) showed a higher expression in amastigotes. Two genes, glucose-6-phosphate dehydrogenase and serine/threonine protein kinase, were found to be similarly expressed in the different biological samples, and were therefore used as controls. The ratios of differentially expressed genes between the stages in this study are high compared to recent microarray studies (Holzer et al. 2006; Leifso et al. 2007; Saxena et al. 2007).

However, the relative differences in gene expression between intracellular amastigotes and promastigotes and between axenics and promastigotes corroborate with the array studies. Although we cannot exclude Leishmania species-related differences (*L. braziliensis* complex versus *L. major* complex), these discrepancies also indicate the fundamental differences between DD analysis and microarray, including the extent of genome coverage, the sensitivity and the risk with DD-analysis to detect several fragments corresponding to the same transcript. Altogether, the interpretation of our DD analysis should be considered with caution. Also, the comparison with results for other species, in biologically different experimental conditions and using other molecular methods, should be made with great prudence.

Having potential infective stage markers in *L. (V.) braziliensis,* our aim in **Chapter 6** was to monitor these putative markers in two representative strains of *L. (V.) braziliensis* (from cutaneous and mucosal origins) and two representative strains of *L. (V.) peruviana* (from cutaneous origin) for a better understanding of the differences in pathogenicity between these two species. To do so, we used quantitative Real-time PCR and the normalization process described by Vandesompele et al. (2002). In the case of the *'meta 1'* gene, we found an increased relative expression level at stationary ("infective") stage (days 4 and 5) in the four strains used here, while the *'opb'* gene expression was variable in the promastigote stages in the four strains analyzed; however, it was found to be up-regulated in the amastigote stages of *L. (V.) braziliensis,* and no signal was detected in *L. (V.) peruviana* at this particular stage. Additionally, the control markers (*'g6p'* and *'stpk'*) showed the expected pattern, homogeneous expression throughout the *in vitro* Leishmania life cycle.
The transcripts corresponding to the infective-insect stage protein (Meta 1 protein) were identified as candidate marker of metayclics. This gene was first identified in *L. (L.) major* by Coulson and Smith (1990) and later in *L. (L.) donovani* and *L. (L.) amazonensis* (Uliana et al. 1999). The gene is predominantly expressed in infective metacyclics of *L. (L.) major* (Nourbakhsh et al. 1996) and over-expression of corresponding Meta 1 protein in *L. (L.) amazonensis*-generated parasites that were more virulent than wildtype organisms *in vivo* (Uliana et al. 1999). Our analyses support the hypothesis that the product of this gene could also be an important virulence factor in parasites of subgenus *Viannia*.

The transcripts corresponding to the Oligopeptidase B protein (*'opb'*) were identified as candidate markers of amastigotes in *L. (V.) braziliensis*. This protein has been shown to be involved in Ca(2+)-signalling during host cell invasion by *Trypanosoma cruzi* (Burleigh et al. 1997, Caler et al. 1998) and in degradation of regulatory peptide hormones in the blood of mammalian hosts in African tryponosomiasis (Troeber et al. 1996). Expression of serine proteases in Leishmania has been proved in previous studies (Colmenares et al. 2001, Silve-Lopez and De Simone 2004, Silva-Lopez et al. 2005, Guedes et al. 2007) and the presence of 'opb' was reported by Ribeiro de Andrade et al. 1998. More recently, Guedes et al. (2007) have amplified the complete open reading frame of obp from *L. (L.) amazonensis* using primers designed for the oligopeptidase B gene from *L. (L.) major*.

Sequencing revealed a protein of 731 amino acids and shares a 90% identity with oligopeptidases of *L. (L.) major* and *L. (L.) infantum*, 84% with *L. (V.) braziliensis*, and approximately 62% identity with *Trypanosoma* peptidases. Guedes et al. (2007) reported the expression of this gene throughout the parasite's life cycle. Their finding was based on RT-PCR (reverse transcription) with promastigotes (at logarithmic and stationary stage) and axenic amastigotes. For our study we used intracellular amastigotes and quantitative PCR and the normalization process with internal control genes. This approach has been shown to be a reliable and sensitive technique for the quantification of mRNA (Ding and Cantor 2004, Vandesompele et al. 2002). Additionally, Holzer et al. (2006) reported minor differences in gene expression between axenic amastigotes and promastigotes, in line with the differences found in our study comparing intracellular amastigotes and promastigotes.

Transcripts corresponding to a serine/threonine protein kinase and glucose-6-phosphate dehydrogenase were likely expressed at the same level in the different biological preparations. A large number of protein kinases (179) were identified in Leishmania (Naula et al. 2005), some of them being exclusively expressed in a given stage (f.i. LmxMKK in the promastigote stage, Wiese et al. 2003). For others (like CRK3), mRNA was found to be constitutively expressed throughout the parasite's life cycle (Wang et al. 1998). A proteomic study of *L. (V.) panamensis* revealed that glucose-6-phosphate dehydrogenase protein was more abundant in amastigotes (Walker et al. 2006). This protein could be another example of post-transcriptional regulation mechanisms which are common in Leishmania and other Trypanosomatids (Stiles et al. 1999).

In summary, by means of molecular techniques and culturing methods we have investigated the evolutionary relationship between Peruvian Leishmania variants. Our data support the hypothesis that *L. (V.) peruviana* did indeed evolve from *L. (V.) braziliensis*. This study has shown that Peruvian Leishmania finds its origin in the Amazonian basin and has crossed the Andes on a defined spot, the Porculla Pass. During the North South colonization of the Pacific slopes of the Andes, the parasites have lost some of the *L. (V.) braziliensis* characteristics showing increasing differences from a karyotype point of view and decreasing pathology (as we have demonstrated by *in vitro* and *in vivo* studies). Possible consequences of this genomic evolution were explored by DD-PCR, which led to the idenfitication of a series of candidate markers of infective stages of *L. (V.) braziliensis*. We have focused further studies on two of them ('*meta 1'* and '*opb'*), and their expression profiles were analyzed in the two species. Metacyclogenesis in the two species can be monitored by the expression of the *'meta 1'* gene, and interestingly, the highest level of *meta 1* expression were measured in the *L. (V.) braziliensis* strain isolated from a mucosal patient. We identified a new marker of amastigote stages, the *'opb'* gene, which is more expressed in amastigotes of *L. (V.) braziliensis* than in the promastigotes stages of the same species, and even though we detected some level of expression at the promastigote stage of *L. (V.) peruviana*, no signal was detected at amastigote stage under our experimental conditions.

CONCLUDING REMARKS

The aim of this study was to explore the contribution of parasite factors to the development of differences in pathology observed in patients infected with *L. (V.) braziliensis* and *L. (V.) peruviana*, using analysis of genomic make-up and stage characteristics. We hypothesized that the *L. (V.) peruviana* descends from the *L. (V.) braziliensis* and acquired its 'peruviana' character during the southward colonization and adaptation of the transmission cycle in the Peruvian Andes. We showed *in vitro* as well as *in vivo* that the *L. (V.) braziliensis* was more infective than *L. (V.) peruviana*. We also found that the *in vitro* infectivity patterns were in line with the geographical gradient of parasite populations. Taking advantage of the differences between the strains, we performed DD analysis, and found two genes that were more expressed in metacyclics (infective insect stage-specific protein, *'meta1'*) and amastigotes (oligopeptidase b, *'opb'*) respectively, in a *L. (V.) braziliensis* reference strain. Further analysis of these genes throughout the *in vitro* Leishmania life cycle enabled us to confirm the use of *'meta1'* gene as metacyclic marker in the two species, while the *'opb'* gene was found to be up-regulated in amastigote stages of *L. (V.) braziliensis;* however, no signal was detected in *L. (V.) peruviana* at this particular stage. Our results, which need to be confirmed by application of these techniques to a larger number of strains, may open new perspectives to understanding the process of speciation in Leishmania and its implications in terms of pathology.

FUTURE PERSPECTIVES

The work presented in this thesis should be viewed as a screening phase. Although transcript levels may suffice to identify potential markers, particular care should be taken when interpreting their biological meaning, complemented by additional studies.

Epidemiological studies should be undertaken along the Peruvian territory base on the BGUs described in this thesis in order to collect additional epidemiological and clinical data of different Leishmania isolates to verify the possible correlation between infectivity and geographical origin of parasite populations with the aim of providing additional evidence confirming the speciation and evolution hypothesis stated for these two species.
It is also important to upgrade *in vitro* as well as *in vivo* models and gather information to extrapolate the data to human infection. These models should mimick the natural course of parasite transmission as far as possible.
Further strains should be analyzed in order to check the level of *'meta 1'* expression in metacyclics, and whether this gene can be used for monitoring metacyclogenesis (for instance to optimize *in vitro* and *in vivo* infections).
Further studies should be developed to confirm our findings in the *'opb'* gene, using more isolates of both species and from different clinical outcomes (i) to verify if this could indeed be a marker for the higher pathogenicity of *L. (V.) braziliensis* (in comparison to *L. (V.) peruviana*) and (ii) to check whether *'opb'* gene expression within *L. (V.) braziliensis*

strains could be related to the different clinical outcomes described for these species (mucosal and cutaneous disease).

The RNA-mediated interference pathway (RNAi) recently found in the genome of *L. (V.) braziliensis* could allow manipulation of gene expression for further analysis of these genes as virulence factors.

In addition, further functional assays with the proteins (Meta 1 and Oligopeptidase b) should also be addressed in order to understand their possible role as virulence factors in Leishmania.

L. (V.) braziliensis/L. (V.) peruviana) hybrids are becoming epidemiologically important emergent genotypes and should also be included in studies to test their *in vitro* as well as *in vivo* infective capacities; in addition, they are interesting organisms to study the gene expression of *'meta1'* and *'opb'*.

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SUMMARY

Leishmania (V.) braziliensis and *L. (V.) peruviana* are the main causative agents of Leishmaniasis in Peru. Even if these two species are very close related at genomic level, they present different clinical outcomes. *L. (V.) baziliensis* is mainly associated with cutaneous lesions and approximately 10 percent of these cases evolve to mucocutaneous lesions; its distribution is restricted to the Amazonian basin. *L. (V.) peruviana*, produces only cutaneous lesions ('uta') and it is distributed along the Andean and inter-Andean valleys. Human factors are important in the development of the different disease outcome; but parasite factors are also playing a key role in the differences observed.

The objective of this thesis was to explore the contribution of parasite factors to the development of the pathology. We aimed to do this by analysis of genomic make up and life stage characteristics.

Chapter 1 reviews the literature on the Leishmania generalities at different levels: diagnostics, treatment and epidemiology, with special emphasis in American leishmaniasis. In addition, we also review the issue of pathogenicity from different point of view: the parasite virulence, the human host and the vector. The molecular biology aspects of the parasite are also reviewed in order to have a background on the actual state of knowledge and studies performed at this level.

In **chapter 2**, we aimed to build up a hypothesis about the evolutionary history of both species, using a rapidly evolving genetic marker. Therefore, chromosomal size of different strains originating from different regions of Peru was processed with a specific algorithm. We hypothesize that *L. (V.) peruviana* would descend from *L. (V.) braziliensis* and would have acquired its 'peruviana' character during the southward colonization and adaptation of the transmission cycle in the Peruvian Andes.

Chapter 3 describes the isolation of Leishmania parasites from the sandfly *Lutzomyia peruensis*. The sandflies were captured in endemic areas at the western slopes of the Peruvian Andes. Dissection of the vector was combined with molecular biological techniques such as PCR. One infected sandfly was found and with a combination of PCR and fragment restriction analysis it was demonstrated that the sandfly carried *L. (V.) peruviana* parasites.

In order to verify if both species showed phenotypic differences in terms of virulence, we developed experimental models **in chapter 4**. Therefore, an *in vitro* and *in vivo* infection model were developed and validated on 2 representative strains of *L. (V.) braziliensis* from cutaneous and mucosal origin and 2 representative strains of *L. (V.) peruviana* from northern and southern Peru respectively. Our models were reproducible and sensitive enough to detect phenotypic differences among strains. We showed *in vitro* as well as *in vivo* that the *L. (V.) braziliensis* was more infective than *L. (V.) peruviana*. We also found that the *in vitro* infectivity patterns were in line with the geographical gradient of parasite populations evidenced previously.

In **chapter 5** we aimed to identify transcripts that might be (i) candidate markers of the infective stages of *L. (V.) braziliensis* (metacyclics and intracellular amastigotes) and (ii) potential controls, i.e. constitutively expressed; these markers might serve as reference for future gene expression profiling studies in *L. (V.) braziliensis* and *L. (V.) peruviana.* Therefore, we applied differential display to screen transcripts originating from different biological stages of a reference *L. (V.) braziliensis* strain. We selected two genes that were more expressed in metacyclics (infective insect stage-specific protein, *'meta1'*) and amastigotes (oligopeptidase b, *'opb'*) respectively. In addition two genes (glucose-6-phosphate dehydrogenase and serine/threonine kinase, *'g6p'* and *'stpk'*, respectively) were shown to be similarly expressed in the different infective stages.

In **chapter 6**, we aimed to monitor the expression of the four genes identified as described in chapter 4 in *L. (V.) braziliensis* and *L. (V.) peruviana*. In this pilot study, we used the 4 reference strains described chapter 3 and followed the expression of the four genes during the *in vitro* life cycle by real-time quantitative PCR. We found, in the case of *'meta1'* gene, an increased relative expression level at stationary ("infective") stage (day 4 and day 5) in the four reference strains. The *'opb'* was found to be over-expressed in amastigote stages of *L. (V.) braziliensis* while a low signal was detected in *L. (V.) peruviana.*

In **conclusion**, we hypothesise that *L. (V.) peruviana* would have evolved from *L. (V.) braziliensis* and that during the North South colonization of the Pacific slopes of the Andes, it would have lost some of the *L. (V.) braziliensis* character: being more and more different from a karyotype point of view, and decreasing its pathogenicity (as here demonstrated by *in vitro* and *in vivo* studies). By DD-PCR, we identified a series of candidate markers of infective stages of *L. (V.) braziliensis* and validated two of them: *'meta 1'* and *'opb'*. These markers were then further explored in the two species. Metacyclogenesis can be detected in both species thanks to the expression of the *'meta 1'* gene, and interestingly, the highest level of *meta 1* expression were measured in the *L. (V.) braziliensis* strain isolated from a mucosal patient. We identified a new marker of amastigote stages, the *'opb'* gene that is more expressed in amastigotes of *L. (V.) braziliensis* than in the promastigotes stages of the same species. Even though, there was some level of expression at promastigote stage of *L. (V.) peruviana*, the expression of this gene was not detected in amastigote stage.

SAMENVATTING

Leismania (V.) braziliensis en *L. (V.) peruviana* zijn de belangrijkste veroorzakers van Leishmaniasis in Peru. Hoewel deze twee species genetisch nauw verwant zijn, verschilt hun klinisch beeld aanzienlijk. *L. (V.) braziliensis* wordt voornamelijk geassocieerd met de cutane vorm van de ziekte. In ongeveer 10 percent van de gevallen ontwikkelt de cutane vorm zich tot een mucocutane vorm. *L. (V.) braziliensis* infecties worden gevonden in het Amazone gebied (oosten van Peru). *L.(V.) peruviana* veroorzaakt alleen cutane zweren (de zogenaamde "uta"). Deze vorm van Leishmaniasis wordt gevonden op de westelijke slopen van de Andes en in de valleien tussen de verschillende Andes massieven. De klinische manifestatie van deze infectie wordt enerzijds bepaald door humane factoren en anderzijds door de eigenschappen van de parasiet zelf.
Het doel van het onderzoek beschreven in dit proefschrift was de bijdrage van de parasiet aan de ontwikkeling van de ziekte te analyseren. Met name richt dit zich op de relatie tussen de expressie van het genoom en de karakteristieken van de levenscyclus van de parasiet.

Hoofdstuk 1 geeft een overzicht van de bestaande literatuur op het gebied van de diagnostiek, behandeling en epidemiologie van Leishmania, met speciale aandacht voor de situatie in Peru. Daarnaast wordt de pathogeniciteit vanuit verschillende invalshoeken belicht: de virulentie van de parasiet enerzijds en de mens en de vector (zandvlieg) als gastheren anderzijds. De moleculaire biologie van parasiet komt ook uitgebreid aan de orde om aldus een beeld te kunnen geven van wat op dit ogenblik bekend is over infecties met Leishmania.

In hoofdstuk 2 hebben we geprobeerd een hypothese op te stellen over de evolutie van de twee Leishmania species gebruik makend van een snel evoluerende genetische marker. Daartoe werd de grootte van chromosomen van verschillende stammen uit verschillende delen van Peru vergeleken aan de hand van een specifiek algoritme. Wij stellen dat *L. (V.) peruviana* afstamt van *L. (V.) braziliensis* en dat dit species de 'peruviana' eigenschappen verworven heeft tijdens de zuidwaartse kolonisatie o.a. door adaptatie van de transmissie cyclus aan de omstandigheden van de Peruviaanse Andes.

Hoofdstuk 3 beschrijft de isolatie van Leishmania parasieten uit *Lutzomyia peruensis* zandvliegen, gevangen in de endemische gebieden aan de voet van de Peruaanse Andes. Dissectie van de vector werd gecombineerd met moleculair biologische technieken zoals PCR. Eén geïnfecteerde zandvlieg werd gevonden en met een combinatie van PCR en restrictie analyse kon aangetoond worden dat het een *L. (V.) peruviana* infectie was.

In hoofdstuk 4 wordt de ontwikkeling beschreven van experimentele in vitro en in vivo modellen, die gebruikt kunnen worden om verschillen in virulentie tussen species resp. stammen aan te tonen. Wij gebruikten daarvoor van beide species twee isolaten: van *L. (V.) braziliensis* een cutaan en een mucocutaan isolaat en van *L. (V.) peruviana* een isolaat uit het noorden en een isolaat uit de zuiden van Peru. Onze modellen bleken voldoende reproduceerbaar en gevoelig om fenotypische verschillen tussen de isolaten aan te tonen.

Zowel in vitro als in vivo bleek *L.(V.) braziliensis* meer infectief te zijn dan *L.(V.) peruviana*. Ook bleek dat de in vitro infectiviteit goed overeenkwam met een eerder vastgestelde gradiënt van noord naar zuid.

In hoofdstuk 5 hebben we geprobeerd een aantal genen te identificeren die 1) als markers bruikbaar zouden zijn voor infectieuze stadia van *L. (V.) braziliensis* (metacyclische parasieten en intra-cellulaire amastigoten), en 2) constitutief tot expressie komen en daardoor kunnen dienen als interne controle. Deze markers zouden in toekomstige studies kunnen dienen als referenties in gen-expressieprofielen van *L.(V.) braziliensis* en *L. (V.) peruviana*. We hebben de 'differential display' methode gebruikt om transcripten van verschillende levenscyclus-stadia van een referentiestam van *L.(V.) braziliensis* te screenen. Uit deze screening zijn twee genen naar voren gekomen die hogere expressie vertoonden in metacyclische parasieten (*meta 1*; een infectiestage-specifiek eiwit) en in amastigoten (*opb*; oligopeptidase-b). Ook werden twee genen, *g6p* (glucose-6-fosfatase) en *stpk* (serine/threonine kinase) geselecteerd die geen expressieverschillen lieten zien in de verschillende infectie stadia.

In hoofdstuk 6 wordt gefocust op het monitoren van de expressie van de vier genen die geïdentificeerd zijn in *L. (V.) peruviana* and *L. (V.) braziliensis* zoals beschreven in hoofdstuk 4. In deze pilotstudie wordt in vier referentiestammen (beschreven in hoofdstuk 3) gevolgd hoe de expressie van deze vier genen verloopt tijdens de verschillende fasen van de levenscyclus. De analyse werd gedaan met behulp van kwantitatieve real-time PCR. We vonden in alle vier de referentiestammen een verhoogde expressie van het *meta 1* gen tijdens de stationaire fase van de infectie (dag 4 en 5 in het experiment). Het *obp* gen vertoonde een verhoogde expressie in de amastigoten van *L. (V.) braziliensis* terwijl er bij *L. (V.) peruviana* een zwakke expressie gevonden werd.

Samenvattend, onze hypothese is dat *L. (V.) peruviana* is geëvolueerd van *L. (V.) braziliensis* en dat gedurende colonisatie van noord naar zuid tussen de Andes en de Stille Oceaan in deze parasieten verschillende, voor *L.(V.) braziliensis* kenmerkende karakteristieken, verloren zijn gegaan. De verschillen komen vooral tot uiting in een afnemende gelijkenis van de karyotypen en een afnemende pathogeniciteit (zoals gedemonstreerd door de *in vitro* en *in vivo* studies). Met behulp van DD-PCR werden een aantal kandidaat-markergenen van het infectieuze stadium van *L. (V.) braziliensis* geïdentificeerd. We hebben twee genen gevalideerd: *meta 1* en *opb*. Deze markers werden gebruikt in verdere studies. De metacyclogenese kan gedetecteerd en gevolgd worden in beide species dank zij de expressie van *meta 1*. Het is opmerkelijk dat in *L. (V.) braziliensis* geïsoleerd van de mucosa van een patiënt de hoogste expressie werd gevonden. Ook werd een nieuwe marker voor het amastigote stadium geïdentificeerd: het gen *opb.* Dit gen komt aanzienlijk meer tot expressie in amastigoten van *L. (V.) braziliensis* dan in het promastigote stadium. Daartegenover staat dat in *L. (V.) peruviana* een laag niveau van expressie in promastigoten gevonden werd terwijl het in het amastigoten niet te detecteren is.

RESUMEN

Leishmania (V.) braziliensis y *L. (V.) peruviana* son los principales agentes causantes de la Leishmaniasis en Perú. A pesar de que estas dos especies están muy relacionadas a nivel genómico, presentan diferente manifestación clínica. *L. (V.) braziliensis* se asocia principalmente con lesiones cutáneas y aproximadamente 10 por ciento de estos casos se convierten en lesiones mucosas; su distribución esta restringida a la Cuenca Amazónica. *L. (V.) peruviana*, solo produce lesiones cutáneas ('uta') y se encuentra distribuida a lo largo de los valles Andinos e Inter-Andinos. Factores humanos son importantes en el desarrollo de las diferentes manifestaciones clínicas de la enfermedad; sin embargo los factores del parásito se encuentran jugando también un rol importante en las diferencias observadas.

El objetivo de esta tesis fue explorar la contribución de los factores del parasito en el desarrollo de la patología. Nuestro objetivo fue hacer un análisis de las características genómicas y de los estadios de vida.

El **capítulo 1** revisa literatura acerca de las generalidades en leishmania a diferentes niveles: diagnóstico, tratamiento y epidemiología, con especial énfasis en Leishmaniasis Americana. Adicionalmente, revisamos el tema de la patogenicidad desde diferentes puntos de vista: virulencia del parásito, el hospedero humano y el vector. Los aspectos de biología molecular del parásito son también revisados para tener información acerca de la situación actual del conocimiento y los estudios realizados a este nivel.

En el **capítulo 2**, nos enfocamos en construir una hipótesis acerca de la evolución de ambas especies, usando un marcador genético de evolución rápida. Por lo tanto, el tamaño de los cromosomas de diferentes especies originadas en diferentes regiones del Perú fue procesado con un algoritmo específico. Nuestra hipótesis es que *L. (V.) peruviana* descendería de *L. (V.) braziliensis* y podría haber adquirido su característica de 'peruviana' durante su colonización hacia el sur adaptando su ciclo de transmisión en los Andes peruanos.

En el **capítulo 3**, nos enfocamos en buscar mosquitos de arena infectados y llevar a cabo la determinación de especies de *Leishmania* combinando la disección del intestino del vector con técnicas de biología molecular. Se encontró una *Lu. peruensis* infectada con *L. (V.) peruviana* identificada usando PCR y PCR-RFLP utilizando dos genes como blanco para la genotipificación molecular.

Con la finalidad de verificar si ambas especies mostraban diferencias fenotípicas en términos de virulencia, desarrollamos modelos experimentales en el **capítulo 4**. Un modelo de infección *in vitro* e *in vivo* fue desarrollado y validado en 2 cepas representativas de *L. (V.) braziliensis* de origen cutáneo y mucoso y 2 cepas representativas de *L. (V.) peruviana* del norte y sur del Perú, respectivamente. Nuestros modelos fueron lo suficientemente reproducibles y sensibles para detectar diferencias fenotípicas entre cepas. Se demostró, tanto *in vitro* como *in vivo*, que *L. (V.) braziliensis* fue mas infectiva que *L. (V.)*

peruviana. También se encontró que los patrones de infección *in vitro* estuvieron en línea con la gradiente geográfica de las poblaciones de parásitos evidenciadas previamente.

En el **capítulo 5**, nuestro objetivo fue identificar transcritos que podían ser (i) marcadores candidatos de los estadios infectivos de *L. (V.) braziliensis* (metacíclicos y amastigotes intracelulares) y (ii) controles potenciales, i.e. expresados constitutivamente; estos marcadores podrían servir como referencia para futuros estudios de perfiles de expresión genética en *L. (V.) braziliensis* y *L. (V.) peruviana.* Por lo tanto, aplicamos exhibición diferencial para buscar transcritos originados a partir de diferentes estadios biológicos de una cepa de referencia de *L. (V.) braziliensis*. Se encontraron dos genes que estuvieron mas expresados en metacíclicos (proteína especifica del estadío infectivo del insecto, *'meta1'*) y en amastigotes (oligopeptidasa b, *'opb'*) respectivamente. Adicionalmente, dos genes (glucosa-6-fosfato deshidrogenasa y serina/treonina kinasa, *'g6p'* y *'stpk'*, respectivamente) mostraron ser expresados de manera similar en los diferentes estadíos infectivos.

En el **capítulo 6**, nos enfocamos en monitorear la expresión de los cuatro genes identificados en el capítulo 4 en *L. (V.) braziliensis* y *L. (V.) peruviana*. En este estudio piloto, utilizamos 4 cepas de referencia descritas en el capítulo 3 y se siguió la expresión de los cuatro genes durante el ciclo de vida *in vitro* utilizando PCR cuantitativo en tiempo real. Se encontró, en el caso del gen *'meta1'*, un nivel incrementado de expresión relativa en el estadío estacionario ("infectivo") (día 4 y día 5) en las cuatro cepas de referencia. La expresión del gen *'opb'* fue mayor en el estadío amastigote de *L. (V.) braziliensis* mientras una señal baja fue detectada en *L. (V.) peruviana.*

En **conclusión**, nuestra hipótesis es que *L. (V.) peruviana* podría haber evolucionado de *L. (V.) braziliensis* y que durante la colonización de Norte a Sur de las pendientes andinas del Pacífico, esta podría haber perdido algunas de sus características de *L. (V.) braziliensis*: siendo mas diferentes desde el punto de vista del cariotipo, disminuyendo su patogenicidad (como se demuestra por los estudios *in vitro* e *in vivo*). Por DD-PCR, identificamos una serie de marcadores candidatos de los estadíos infectivos de *L. (V.) braziliensis* y validamos dos de ellos: *'meta 1'* y *'opb'*. Estos marcadores fueron posteriormente estudiados en dos especies. La metacyclogénesis puede ser detectado en ambas especies gracias a la expresión del gen *'meta 1'*, e interesantemente, el nivel mas alto de expresión de *meta 1* fue medido en la cepa de *L. (V.) braziliensis* aislada de un paciente mucoso. Identificamos un nuevo marcador del estadío amastigote, el gen *'opb'* que es mas expresado en amastigotes de *L. (V.) braziliensis* que en promastigotes de la misma especie. A pesar que, hubo algún nivel de expresión en el estadío promastigote de *L. (V.) peruviana*, la expresión de este gen no fue detectada en el estadío amastigote.

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